THE ROLE OF MIG6 IN PANCREAS DEVELOPMENT AND DIABETES

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Cellular and Integrative Physiology,
Indiana University

October 2018

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

To my parents, who worked hard and sacrificed more than I can imagine to encourage, support, and provide for me throughout my education, and who have always loved me unconditionally.

ACKNOWLEDGEMENTS

I was fortunate and am greatly appreciative to receive my pre-doctoral fellowship award from the American Heart Association to push my studies and career forward. Additionally, I would like to take this opportunity to thank several people without whom this would not have been possible. First, my mentor, Dr. Patrick Fueger. During my final rotation, Patrick announced he would be accepting other students, and he was gracious enough to take me as his mentee. Patrick designed his lab to be an open, supportive environment and our research interests aligned: we have been tackling diabetes together for over 5 years, on two fronts. He has taught me so much about being a researcher and a scientist. Not only is his research intellectually stimulating and relevant, his mentorship is without comparison. Patrick has been a pillar of support throughout my journey – present and encouraging during victories and defeats, both professional and personal. I could always talk to him honestly about what I was struggling with, and he was always understanding and caring. He is down to earth, funny, confident, and driven, so I strive to be all of these things as well. I am extremely fortunate to know Patrick and have him as my mentor – *Thank you!*

I would also like to thank my research advisory committee: Dr. Fred Pavalko, Dr. Ryan Anderson, Dr. Laura Haneline, and Dr. Charlie Dong. When Patrick moved to City of Hope in California, I chose to remain in Indianapolis to finish my research. Everyone was so supportive and patient during this transition. Dr. Fred Pavalko adopted me under his supervision and supported me as needed while I pursued my research from afar. Fred would always remind me that it does no good to worry, and encouraged me through every

endeavor. Dr. Ryan Anderson became a close mentor and guided me through a separate and unique project, which proved to be an incredibly fulfilling experience for me, both educationally and professionally.

Wise input from several mentors and peers has pushed my projects forward and my career path into higher clarity. Specifically, I will highlight Dr. Debbie Thurmond, Dr. Andrew Lutkewitte, Dr. Angelina Hernandez-Carretero, Paul Miller, and Abass Conteh, who have been encouraging, friendly faces in times of uncertainty.

I want to acknowledge my family and friends who have been with me through the program, trying to keep me sane as I learned how to balance research life and life life. Rochelle Frankson is my dear friend and motivator – she is always positive and encouraging, and someone I could rely on to gently guide me to the truth. Kadijah Porter (M.D. to be, hollaaaa) is an amazingly hard-working friend and it has been a joy to watch her persevere and thrive. Samantha Huffman, my longest friend, was always there for me and always supportive through every struggle. My sisters, Bella and Samantha, and my parents, Don and Dianne Barriger, have always backed me in whatever I do, and have endured some long stints of silence during these few years. My in-laws, Sheri, Myron, Myron II, and Josie, encouraged me to grow even when I believed I was done growing. Thank you all for your encouragement, always letting me know how proud you are, and helping me to step back and see that what I do is actually pretty cool.

Next, my dear husband, Marcus. *Thank you.* Thank you for all your love, support, patience, and confidence. There were countless times you talked me down from a hysterical moment of self-doubt or a panic-stricken bid to give up. You encouraged me to be open with my peers and mentors about the struggles that I was going through and not

to be afraid to admit that I was overwhelmed at times. You are logical and your thoughts are always collected – a complement to my sometimes irrational and haphazard thoughts. You have always pushed me to do both what I love to do and what makes use of my talents and skills. You gave me freedom to choose what I want to do, and you made me choose it for myself, not anyone else. There is a considerable difference between the person who started this program and the one who will defend this thesis, and that is, in large part, due to you. Even *I* finally notice the change, and there will be more to come. As we enter into our next adventure, you (and Wale) will remind me that, like any growth, you can't be ready for it – it's growth. It's going to be new, you're going to have a new life, and you're going to be a new person. And so, we will keep growing and changing. I love you.

And finally, all the praise to God. Every accomplishment during my journey is and will be credited to Him. I am just here to do my best with the gifts and time He has allotted to me.

Kimberley Mei Ling El

THE ROLE OF MIG6 IN PANCREAS DEVELOPMENT

AND DIABETES

Diabetes occurs as a result of the failure of pancreatic insulin-producing β cells. The preservation or renewal of β cells is a strategy that can prevent diabetes by targeted manipulation of mechanisms associated with autoimmune β cell destruction or β cell regeneration. ErbB signaling, specifically epidermal growth factor receptor (EGFR) signaling, is associated with cell survival, growth, and proliferation. Thus, we investigated the role of the ErbB inhibitor, mitogen-inducible gene 6 (*mig6*), in pancreas development and in the progression to diabetes. Using morpholino knockdown in a zebrafish model of development, we discovered that *mig6* is required for the generation of α and β cells as well as the formation of the exocrine pancreas. We suspect that the loss of *mig6* function causes premature differentiation of ductal progenitor cells, and acts as a switch between progenitor differentiation and endocrine transdifferentiation. Furthermore, we established a pancreas-specific *mig6* knockout mouse that maintained glucose tolerance and had a higher β cell mass after chemically-induced β cell injury by way of increased β cell proliferation. Our data suggests that *mig6* is required during pancreas development and may be employed as a switch to direct the production of new β cells, but that during adulthood, it is detrimental to the recovery of β cell mass, making it a therapeutic target for β cell preservation after the onset of diabetes.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Arx	Aristaless Related Homeobox
Ascl1	Achaete-scute family BHLH transcription factor 1
BG	Blood glucose
Cas9	CRISPR-associated protein 9
CRIB	Cdc42- and Rac-interactive binding
CRISPR	Clustered regularly interspaced short palindromic repeats
CS	Carnegie stage
DIG	Dioxygenin
DPTA/NO	Dipropylenetriamine NONOate
EGF	Epidermal growth factor
EGFR	EGF receptor
EPD	Extrapancreatic duct
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1 and 2
ESC	Embryonic stem cell
Fabp10	Fatty acid binding protein 10
fISH	Fluorescent *in situ* hybridization
Foxa2	Forkhead box a2
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide 1

GRB2	Growth factor receptor bound protein 2
gRNA	Guide RNA
GSIS	Glucose stimulated insulin secretion
H2BRFP	Histone 2B red fluorescent protein
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HTSM	Human tracheal smooth muscle
IA-2	Islet antigen 2
ICA	Islet cell antibody
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1-beta
IL6	Interleukin 6
iNOS	Inducible nitric oxide synthase
ISH	*In situ* hybridization
L-NMMA	N^G-Monomethyl-L-arginine
LRC	Label retaining cell
LRIG1	Leucine rich repeats and immunoglobulin like domains 1
MafA	MAF BZIP transcription factor A
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
Mig6	Mitogen-inducible gene 6
MLD	Multiple low-dose
MO	Morpholino

MTZ	Metronidazole
NeuroD	Neuronal differentiation
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ngn3	Neurogenin 3
Nkx2.2	NK2 homeobox 2
Nkx6.1	NK6 homeobox 1
NO	Nitric oxide
NOD	Non-obese diabetic
NTR	Nitroreductase
Pax6	Paired box 6
PCR	Polymerase chain reaction
PDX-1	Pancreatic and duodenal homeobox-1
PI3K	Phosphoinositide 3 kinase
PKO	Pancreas-specific knock out
PLC-γ	Phospholipase C gamma
Ptf1a	Pancreatic transcription factor 1a
PTU	Phenylthiouria
ROS	Reactive oxygen species
SD	Standard deviation
SEM	Standard error mean
SFK	Src family of protein tyrosine Kinases
SH2	Src homology 2
SH3	Src homology 3

SOCS4	Suppressor of cytokine signaling 4
SOCS5	Suppressor of cytokine signaling 5
Sox9	SRY (sex determining region Y)-box 9
Src	Proto-oncogene tyrosine-protein kinase Src
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
ZnT8	Zinc transporter 8

CHAPTER 1. INTRODUCTION

1.1 Diabetes

Diabetes is a metabolic disorder of the endocrine pancreas and peripheral target tissues. It manifests as abnormally elevated blood glucose levels (hyperglycemia), with symptoms such as increased thirst and hunger, frequent urination, and numbness in the extremities. Hyperglycemia is detrimental to all other systems, especially the cardiovascular and renal systems, and prolonged exposure of these systems to hyperglycemia can induce coma and cause death.

Epidemiology and diagnosis of Type 1 Diabetes

Diabetes is one of the world's most common and fastest growing non-communicable diseases. Globally, an estimated 422 million people were living with diabetes in 2014 [1], and in the United States, 23.1 million people were diagnosed with diabetes [2]. Both Type 1 Diabetes (T1D) and Type 2 Diabetes (T2D) are defined by marked deficiencies in the production or utilization of insulin, the critical blood glucose (BG)-lowering hormone required to maintain glucose homeostasis. T2D occurs when the body is unable to effectively use the insulin that it produces (i.e., relative insulin insufficiency), whereas T1D (previously known as juvenile, insulin-dependent, or childhood onset diabetes) is when the pancreas fails to make enough (or any) insulin (i.e., absolute insulin insufficiency). T1D accounts for 5-10% of cases of diabetes. This dissertation will focus on T1D.

The symptoms of diabetes include increased thirst and hunger, frequent urination, and increased tiredness. Clinically, a patient with prolonged exposure to hyperglycemia

presents with hyperphagia (hunger), polydipsia (frequent thirst), polyuria (frequent urination), weight loss, and possibly even diabetic ketoacidosis. Diabetes is diagnosed by measuring fasting BG levels or based on the HbA1c test, which measures glycated hemoglobin over 3 months. The criteria for diabetes diagnosis are any of the following: HbA1c ≥6.5%, fasting BG ≥126 mg/dL, a 2h-postprandial BG ≥200 mg/dL, or, in a patient with clinical symptoms, a random BG ≥200 mg/dL [2]. After diabetes diagnosis, physicians will run more complex diagnostic tests to determine the type of diabetes.

T1D can be distinguished by the presence of markers like C-peptide, autoantibodies, and ketones. During insulin synthesis, preproinsulin is translated into proinsulin and processed through the endoplasmic reticulum (ER), where it is modified and packaged for transport. Proinsulin is packaged into secretory granules where proteases in the granule cleave the protein in two places to excise C-peptide, leaving the finished insulin product: two peptide chains linked by two disulfide bonds. Both the final insulin product and C-peptide are released into the blood stream. Thus, C-peptide is a marker for insulin production. Unlike insulin, C-peptide is not removed by the liver and is eventually excreted into the urine, providing a quantitative measure for insulin secretion. For this reason, C-peptide, rather than insulin, levels are commonly used to differentiate T1D from T2D, although late stage T2D is also characterized by decreased C-peptide levels.

In addition to this marker, the presence of autoantibodies can confirm T1D diagnosis. Autoantibodies are produced by the B cells of the immune system when it fails to distinguish between ‘self’ and ‘non-self.’ Autoimmune markers for T1D include islet cell autoantibodies (ICA), insulin, GAD/GAD65, tyrosine phosphatases IA-2 and IA-2β,

and zinc transporter 8 (ZnT8) [3-5]. These autoantibodies can be present alone or in combination, but their correlation is evident, as 93% of young adults with T1D test positive for one or more of these autoantibody diagnostic markers [6].

Finally, the presence of ketones in the urine is indicative of T1D. Without insulin, the cellular uptake of glucose from the bloodstream is markedly reduced. As a result, cells such as myocytes and hepatocytes metabolize fat and protein into fatty acids (FAs) and amino acids, respectively. In addition, the liver produces *more* glucose because the cells are not receiving the glucose needed to function. The excess glucose signals further FA production. The liver then converts FAs into ketones as an alternate energy source [7]. Increased glucose and ketones in the blood increases the acidity of the blood (i.e., diabetic ketoacidosis) and can prove fatal if untreated.

Etiology of T1D

Whereas the rising incidence of T2D correlates with the rise in obesity [8], the rise in T1D incidence [9] remains inexplicable. T1D is characterized by the failure of the β cell to produce insulin. Suspected etiology of T1D is diverse and ranges from genetic predisposition to viral infections and sanitation practices, but progression of the disease is likely a concerted effort and, mechanistically, occurs differently in each person based on their (epi)genetic milieu [2, 6]. However, definitive findings demonstrate that T1D is autoimmune-mediated, meaning, for reasons undefined, the body initiates an immune response to endogenous insulin and the pancreatic β cells that produce insulin, resulting in insulin deficiency and hyperglycemia [10].

Of particular interest is the genetic contribution to the onset of T1D. Although over 80% of T1D cases occur in individuals with no apparent family history [11], familial

studies in first degree relatives of patients with T1D demonstrate that the risk of developing diabetes averages 6% in offspring, 5% in siblings, and 50% in identical twins [12-14]. Human chromosome 6 contains human leukocyte antigen (HLA) genes that are involved in the immune system's ability to distinguish self from non-self, and these genes are directly correlated to T1D. Incidence of T1D is the highest in those expressing HLA-DR4 and/or HLA-DR3 alleles and certain HLA-DQ alleles as illustrated in **Figure 1-1** [6, 15].

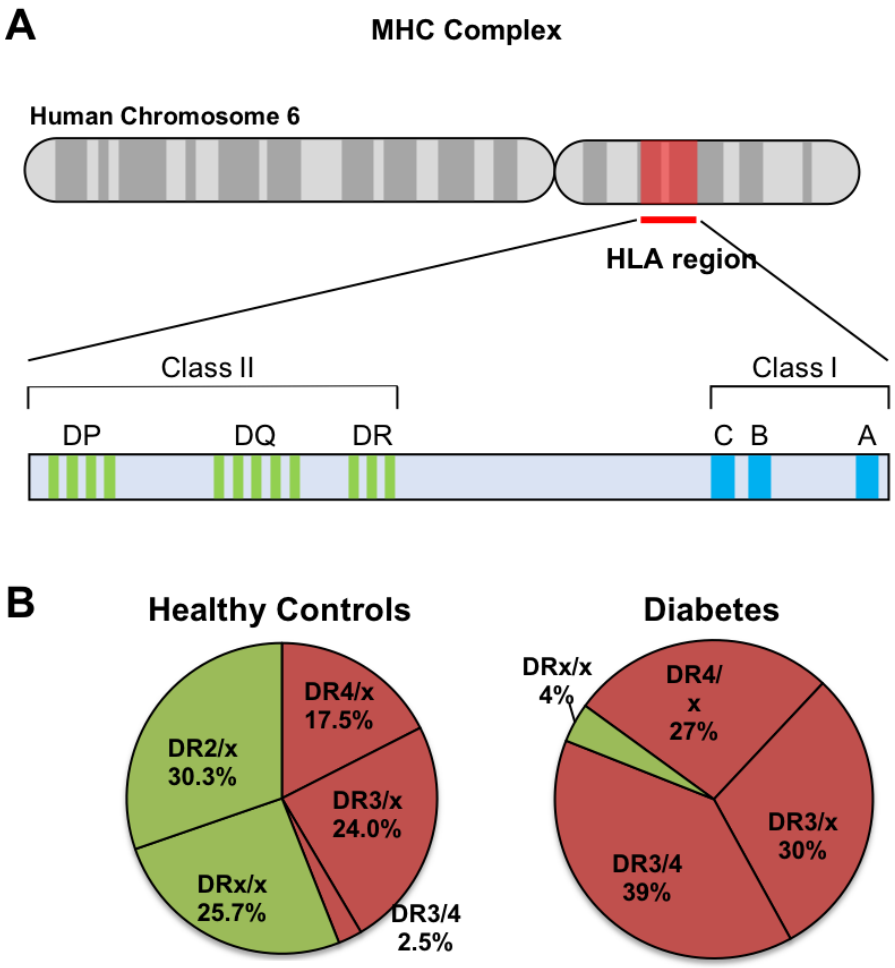


Figure 1-1. Genetic susceptibility to diabetes. (**A**) Human chromosome 6 contains HLA genes, and (**B**) population studies reveal association of HLA genes with T1D.

Only a 50% concordance between identical twins would suggest that there are additive factors to genetic predisposition that are contributing to diabetes onset and progression. In fact, external environmental factors are likely to contribute to T1D. Candidates for environmental factors include viral infection, geographical region, and sanitation practices [6, 16-18]. Viruses are widely suspected of being environmental triggers. Enteroviruses are thought to contribute to the pathogenesis of T1D by presenting autoantigens and pro-inflammatory cytokines – at least, this theory has dominated the field. Enteroviruses are suspected to trigger T1D by molecular mimicry, islet inflammation, or inhibition of ductal cell differentiation [19-22]. Geographical location may also influence T1D onset. There is evidence of a north-south gradient of disease incidence with the highest rate of T1D development in Northern Europe with decreasing incidence in southern or tropical climates [23-25]. The hygiene hypothesis to explain the rise in T1D also has merit. T1D is more prevalent in highly developed nations that have increased sanitation [26, 27]. Additionally, in the experimental non-obese diabetes (NOD) mice (the gold standard mouse model of T1D) bred under pathogen-free conditions have the highest rate of diabetes development, compared to animals bred in a conventional environment [28-30].

Following onset of T1D, a series of immunological attacks occur where β cell ablation continues. The attack on β cells is largely due to recruitment of autoreactive T cells [11] as well as possible progressive destruction of the pancreatic islet depending on cyclical patterns of exposure to islet autoantibodies [31, 32]. Due to the cyclical nature of antigen presentation, it is suggested that T1D is a relapsing-remitting disease [33], similar to designations given to other autoimmune diseases, such as multiple sclerosis or

rheumatoid arthritis. The characteristic recurrent episodes of inflammation describe the progressive destruction of the pancreatic islets in T1D [34].

Progression to T1D

β cell destruction in T1D progresses over time and often no symptoms present until critical β cell mass is reached. β cell mass is the relative percentage of β cell area multiplied by total pancreas mass. β cell mass is lost in a stair-step manner [33, 35]. Changes in β cell mass during T1D progression are summarized in **Figure 1-2**. Persons genetically susceptible to T1D are exposed to one or more environmental factors that initiate an autoimmune attack, resulting in a loss of β cell mass, until symptoms present and diabetes is diagnosed. During the period between diagnosis and complete β cell destruction, termed the honeymoon phase [36], there is residual β cell mass. Current research means to take advantage of the honeymoon period as an intervention window to introduce insulinotropic agents or β cell restoration methods to prolong or increase endogenous β cell function [37].

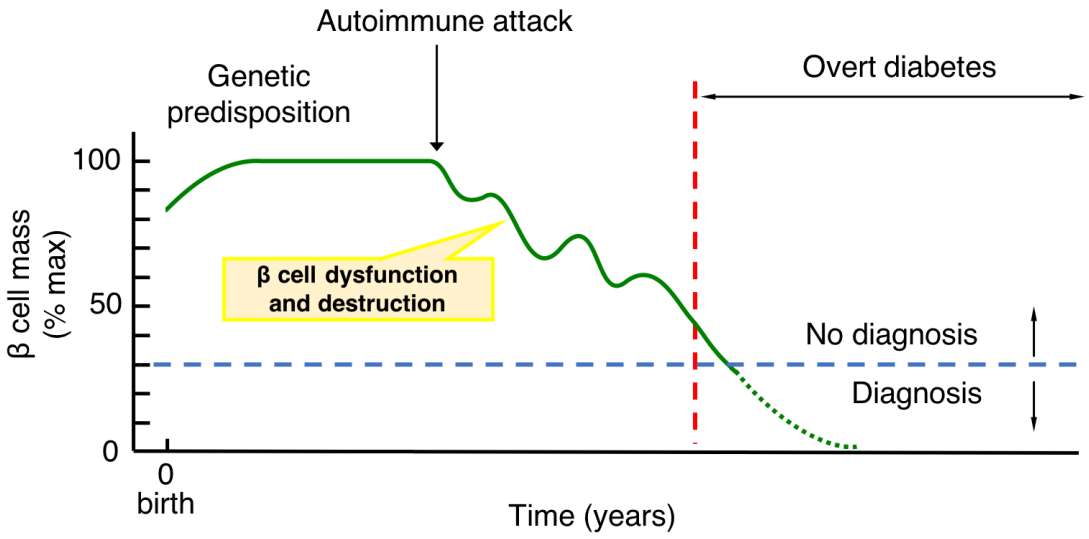


Figure 1-2. Natural history of Type 1 Diabetes *adapted from [35]*.

Management and current therapies

Current management strategies for T1D are intensive insulin therapy and β cell replacement via transplantation. Human islet transplantation has been extensively assessed as a means to cure T1D, with outcomes of 3- to 5- year-long insulin independence in almost half of transplanted patients [38]. However, translation of this procedure to routine clinical treatment has been restricted by donor organ shortage and the need for long-term immunosuppression drugs [39].

The majority of patients with T1D manage blood glucose levels by administration of exogenous insulin, an arduous and expensive endeavor over the lifetime of a patient. Patients often have bouts of burnout, which may affect adherence to treatment strategies established between the patient and their healthcare providers. It is pertinent to identify alternative treatment strategies. Studies have largely focused on the immune response, but, to date, immunological treatments have been largely ineffective [40]. Instead, cellular therapies are a growing alternative that can promote β cell proliferation, survival, and recovery [41]. These therapies may be useful in restoring β cell mass during the honeymoon period to delay insulin dependence.

1.2 Pancreas development and function

Key elements to the restoration of β cell mass may be uncovered by understanding the origins and cross-talk of the pancreatic endocrine cells that regulate glucose homeostasis. To elucidate these origins, we explore genes in the context of pancreas development and organogenesis. Work in this area may reveal transcription factors that control cell fate, and can be utilized for *in vitro* pancreatic differentiation.

Transcription factors decide endocrine cell commitment

The pancreas forms from two buds – the dorsal and ventral buds – that are formed from the distal foregut endoderm. At the final stage of pancreas specification, the different cell types in the pancreas can be distinguished by their transcription factor expression profiles. Human embryogenesis (fertilization until 8 weeks post conception [wpc]) is divided into 23 Carnegie Stages (CS). As presented in **Figure 1-3**, the various cells types that arise in the human pancreas have distinct cell lineages. These transcription factors can be used to differentiate cells *in vitro* to create β cells or β-like cells for transplant or drug screening [42-44]. Human pancreatogenesis is similar to mammalian models of pancreas development, but transcription factor expression varies.

Pancreas and duodenum homeobox 1 (*Pdx1*) and Pancreas transcription factor 1a (*Ptf1a*) are the main initiators of pancreas differentiation. *Pdx1* is expressed throughout pancreas development and its expression does not wane until maturation of late progenitor cells into duct or endocrine cells [45, 46]. In fact, knock out of *Pdx1* results in absence of pancreatic tissues [47] and impeded pancreatic progenitor expansion, but β-like and α-like cells persist [48], indicating that *Pdx1* is not necessary for early differentiation of endocrine cells. However, *Pdx1* is crucial for β cell function and maintenance of β cell maturity. *Pdx1* expression is progressively confined to the endocrine β cell [49] and, in mature β cells, reduction of *Pdx1* depletes insulin and induces glucose intolerance [50], suggesting that *Pdx1* is required to maintain normal β cell function.

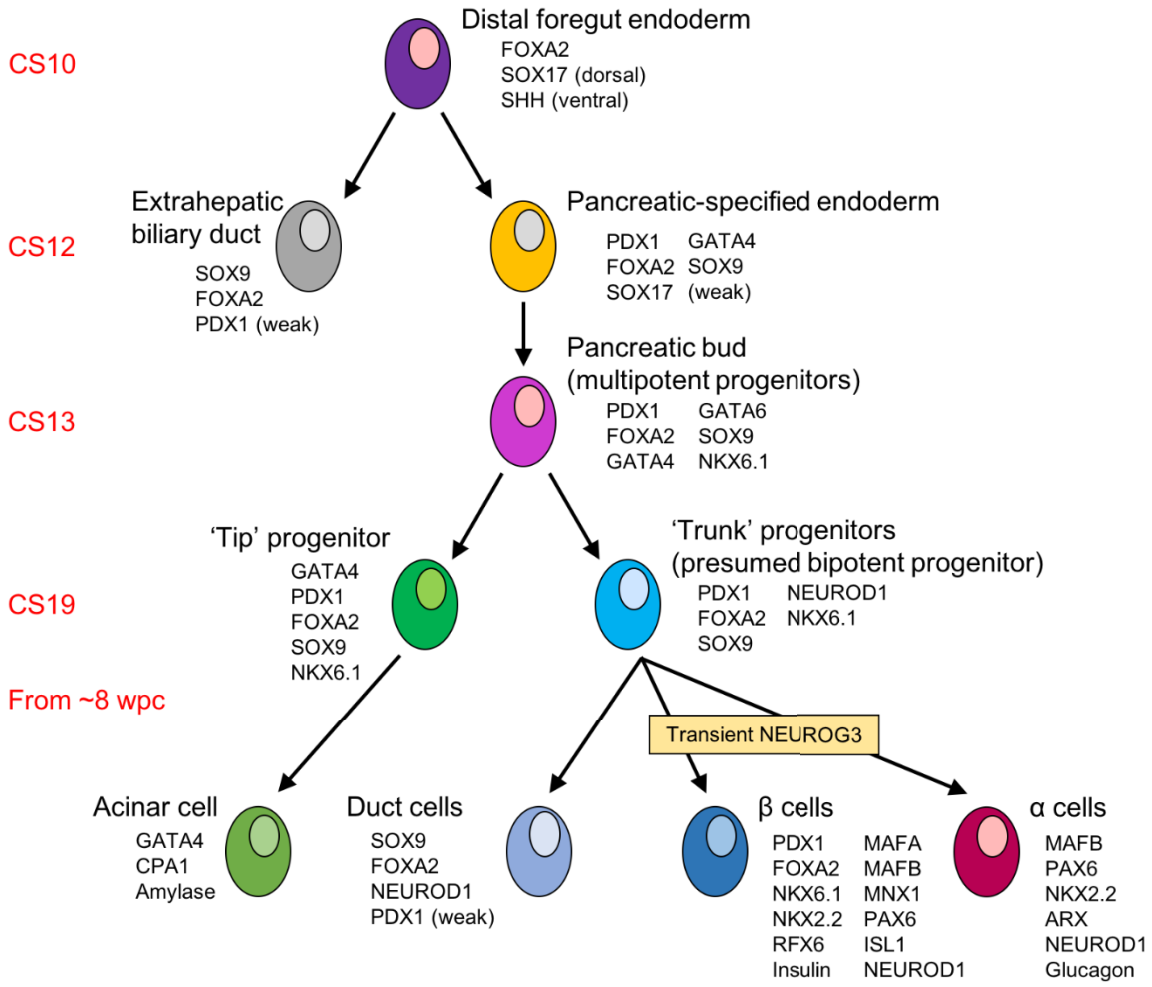


Figure 1-3. Transcription factors involved in cell specification during human pancreas development. Modified from [42].

Like *Pdx1*, *Ptf1a* is another transcriptional activator required for the determination of the fates of all pancreatic cells. The inactivation of *Ptf1a* alters the character of pancreatic progenitors and evidence reveals that in undifferentiated *Pdx1*-expressing progenitors, co-expression of *Ptf1a* is required for development of a pancreatic fate [51]. Further evidence of a synergistic relationship with *Pdx1* was provided in reports of a functional *Ptf1a*-binding site on the *Pdx1* promoter where *Ptf1a*

transactivates the *Pdx1* gene [52, 53], suggesting that the mutual actions of *Ptf1a* and *Pdx1* are crucial for early foregut progenitors to acquire a pancreatic cell fate [54].

With time, *Pdx1* and *Ptf1a* are restricted to the acinar progenitor cells, while *Sox9* and *Nkx6.1* are expressed in the bipotent progenitors of the trunk giving rise to both duct or endocrine cells [55]. Eventually, some trunk progenitors will increase expression of Neurogenin3 (*Ngn3*), resulting in endocrine cell differentiation. Loss of *Ngn3* results in complete absence of α, β, δ, and pancreatic polypeptide endocrine cells [56], supporting the governing role of *Ngn3* in endocrine cell specification. Endocrine cell fate in the pancreas is also decided by *MafA*, which is increasingly expressed over the course of pancreas development. Loss of *MafA* results in impaired glucose stimulated insulin secretion (GSIS), abnormal islet morphology, and diabetes [57]. Together, overexpression of *Pdx1*, *Ngn3*, and *MafA* causes pancreatic exocrine progenitors to become β-like cells.

Endocrine hormones regulate endocrine cell development

Differentiated endocrine cells feedback to progenitors to facilitate cell differentiation. Studies in the glucagon-producing α cells have indicated that knock down of the *glucagon* gene in zebrafish facilitated α to β cell transdifferentiation, suggesting that *glucagon* gene products act as permissive signals to disrupt α cell stability [58]. In fact, GLP-1 (an incretin hormone derived from the pre-proglucagon gene and secreted primarily by the intestinal enteroendocrine L cells) is implicated in the protection and promotion of β cell regeneration through paracrine signaling between adjacent α cells and injured β cells [59]. Although the exact mechanism of β to α cell cross talk is not well defined, α cell proliferation is proposed to contribute to α cell hyperplasia observed in β

cell injury models [60]. This expansion of α cells provides an enlarged pool for α to β cell transdifferentiation.

Table 1-1. Pancreatic cells and their physiological functions

Pancreas type	Cell	Secretes	Physiological Function
Endocrine	β	insulin, amylin	lowers blood glucose
	α	glucagon	raises blood glucose
	δ	somatostatin	decreases GI functions, inhibits glucagon and insulin secretion*
	F (PP)	pancreatic polypeptide	reduces gastric acid secretion, increase intestinal nutrient transit times, inhibits postprandial exocrine pancreas secretion
	ε	ghrelin	enhances GSIS*, stimulates hunger
Exocrine	acinar	digestive enzymes in H^+-rich fluid	nutrient breakdown, induces alkaline fluid production by duct cells
	duct	alkaline buffer fluid	neutralizes both gastric acid entering duodenum and acidic acinar digestive enzymes, and facilitates fluid movement to flush pancreatic enzymes into duodenum

*supposed function

Function of endocrine versus exocrine pancreas

Lineage tracing experiments in rodents have demonstrated that *Pdx1*-expressing progenitors give rise to all three pancreatic cell-types: exocrine, endocrine, and duct [61, 62]. The exocrine pancreas secretes enzymes into the intestine whereas the endocrine pancreas secretes hormones into the bloodstream. **Table 1-1** lists the pancreatic endocrine and exocrine cells and their physiological functions. Endocrine cells are sensors and regulators of glucose homeostasis and are responsible for maintaining blood glucose within normal physiological levels (in humans, 70-130 mg/dL). Glucose homeostasis is mainly regulated by the actions of two opposing cell types: β cells and α cells. Insulin

decreases blood glucose levels by promoting glucose uptake into peripheral tissues. Glucagon increases blood glucose by stimulating breakdown of glycogen to glucose in the liver and activating gluconeogenesis [63]. Insulin also suppresses the production of glucose by the liver. Glucose homeostasis is a precise physiological balance that, when disrupted, causes unregulated blood glucose and diabetes.

1.3 Autoimmunity and β cell destruction

Autoimmune destruction of the pancreatic insulin-producing β cells embodies T1D. As briefly mentioned above, progression to overt T1D involves a myriad of circumstances to promote β cell necrosis and apoptosis.

Autoimmune pathology

In this section, the components of the immune system and their effect on β cell mass are explained in more detail. β cell destruction in T1D begins with an environmental trigger that activates autoimmunity. Normally, through a process known as tolerance, the immune system is trained to identify and ignore the body's own cells (i.e., self), but must concurrently identify and fight foreign cells that pose a threat (i.e., non-self). Foreign antigens are enveloped and presented on the surface of professional antigen-presenting cells (such as dendritic cells and B cells) by major histocompatibility complexes (MHC) class II molecules, initiating an immune response to the foreign antigen [64]. When the immune system fails to distinguish 'self' from 'non-self,' it produces autoantibodies to specific 'self' antigens. Testing positive for all three autoantibodies to GAD65, IA-2, and insulin is highly predictive of diagnosis of T1D [3,

5]. Presentation of GAD65, IA-2, and insulin causes insulitis, whereby immune cells are recruited to the islet, infiltrate it, and attack the β cells.

In T1D, it is hypothesized that tolerance to self-antigens expressed in the islets fails to develop or persist [65]. The previously mentioned NOD mouse is frequently used as a model of T1D because it spontaneously develops diabetes. The implications of immune tolerance on autoimmune diabetes are exemplified in the following study demonstrating that in *proinsulin* conditional knock out NOD mice, enhancing tolerance to *proinsulin* during the period from gestation to weaning was sufficient to protect against diabetes in the long-term [66]. Although the exact mechanism of T cell antigen presentation is poorly defined, it is widely supported that differential affinity for thymic-presented self-antigens determines whether potential auto-reactive T cells are deleted or converted to regulatory T cells (Tregs) during thymus development [66-69]. An alternative explanation that supplements the T cell receptor affinity hypothesis has slowly gained popularity as a complement to the affinity model. This alternative hypothesis was first proposed in the 1980s [70] and presented the idea that mutations in islet antigens may prevent the clonal deletion of autoreactive T cells in the thymus, and evidence has been collected to support this idea [71]. Unfortunately, immunological therapies targeting immune suppression and tolerance have yet to translate into effective long-term therapies for humans [40]. Alternatively, investigations into cellular therapies have promising results and may be administered in combination with immunological therapies or other cellular therapies [72]. Therefore, scientists continue to unravel the mechanisms of autoimmunity to develop better cellular therapies.

Cytokine signaling

One mechanism of focus concerning immune cells is the release of pro-inflammatory cytokines into the islet by $CD4^+$ and $CD8^+$ autoreactive T cells, which causes β cell death [67, 73-75]. Cytokine signaling is well known to contribute to β cell death, but the cytokines IL-1β, TNF-α, and IFN-γ that are released by T cells and macrophages have a more finessed role in the progression to T1D. Pro-inflammatory cytokines are crucial to development of diabetes. For example, neutralization of IL-1β, IFN-γ, IL-6, and TNF-α can inhibit the spontaneous development of diabetes in NOD mice [76].

Cytokines have a variety of effects on β cells. They sensitize β cells to apoptosis by expressing pro-apoptotic proteins [77]. They facilitate the autoimmune response and attack by triggering suicidal secretion of chemokines by β cells, resulting in continuous recruitment of autoreactive T cells [78]. Lastly, cytokines cause direct stress to the β cell. These effects ultimately activate the cell's death machinery. However, any pro-inflammatory cytokine alone has somewhat limited effects on cell stress or β cell death; however, in combination, they have very strong effects that induce enough stress to lead to cell death [79]. This response is probably mediated by nitric oxide (NO production). NO production can be measured by nitrite levels in cytokine-treated human islets. Nitrite production in human islets increased in conditions where islets were exposed to multiple cytokines, with the most nitrite produced when islets were treated with the combination of pro-inflammatory cytokines, IL-1β, TNF-α, and IFN-γ [80].

Immune-mediated loss of functional β cell mass

In response to the rise in cytokine-facilitated cell death, the islet compensates by increasing β cell proliferation. However, eventually the β cells become overwhelmed, the islet degranulates and compensatory β cell function ceases, impairing glucose tolerance, decreasing insulin secretion, and reducing overall β cell mass [37]. Functional β cell mass is regulated by the balance between β cell expansion (e.g., proliferation, transdifferentiation, and neogenesis) and destruction (e.g., death and dedifferentiation).

Autoreactive immune cells in diabetes persist after diagnosis and management of symptoms. This concept was implied in a series of clinical trials in the 1980s revealing that cyclosporin therapy led to remission of T1D, but only when administered within months of disease onset when there was residual β cell mass to maintain glucose homeostasis [81-83]. The toxicity of drugs such as cyclosporin prevented its approved use for the treatment of T1D. Consequently, when cyclosporin therapy ceased, T1D relapsed rapidly [84, 85]. These studies revealed that (safer and more targeted) immune suppression could be an effective treatment for T1D, but immunotherapies developed since have proven ineffective in the long-term.

1.4 Mitogenic signal control of functional β cell mass

Cellular therapies are gaining attention as promising alternatives to ineffective immunological treatments. Mitogenic signaling induces mitosis. Mitogens like insulin, glucose, incretins, insulin-like growth factor, epidermal growth factor (EGF), and HGF, have been demonstrated to increase β cell expansion [86-89]. However, there is a gap in the understanding of how mitogenic signaling is regulated.

ErbB receptor tyrosine kinase signaling

The ErbB receptor family has four members: the EGF receptor (EGFR, known as ErbB1), ErbB2, ErbB3, and ErbB4. Generally, each receptor consists of an extracellular binding domain (except ErbB2), a transmembrane domain, and an intracellular kinase domain (except ErbB3) [90]. Upon ligand binding, the ErbB receptors either homo- or heterodimerize to active the intracellular domain, leading to recruitment of adapter proteins to activate ERK and PI3K signaling [91].

EGF and EGFR

Specifically, EGFR signaling controls key cell fate programs, including cell survival, proliferation, and differentiation [92]. EGFR and its ligand, EGF, have been implicated in increased β cell mass and protection against diabetes. EGFR is also essential for normal pancreatic development and postnatal β cell proliferation [93-96]. Mice with constitutively active EGFR treated with the β cell toxin streptozotocin (STZ) were protected from hyperglycemia, had a higher survival rate, and higher β cell mass compared to control animals [97]. Conversely, mice expressing a dominant-negative EGFR developed diabetes within 2 weeks of birth, had lower insulin levels, and had fewer islets compared to control mice [95].

As a cellular therapy, EGF and gastrin in combination dampened hyperglycemia in STZ-treated rats [98]. In addition, administration of EGF and gastrin increased β cell mass and reversed hyperglycemia in NOD mice [99]. However, this therapy has yet to translate into a viable treatment for humans [100]. EGF therapy also raises concerns around specificity of β cell expansion, as some non-specific signals could promote

inadvertent growth of other cells. This unwarranted effect can be avoided by indirectly targeting EGFR through targeting its negative regulators.

Inducible feedback inhibition of EGFR

The EGFR signaling cascade is one of the most investigated molecular pathways in regulation of cell fate. Thus, to ensure proper signaling, EGFR activity is tightly controlled by a number of negative regulators, as a built-in security feature [101, 102]. Of these various regulatory programs, EGFR is subject to four mechanisms of inducible feedback inhibition by leucine-rich immunoglobulin-like domains protein 1 (LRIG1), suppressor of cytokine signaling 4 and 5 (SOCS4 and SOCS5), and mitogen-inducible gene 6 (Mig6) [103]. These inhibitors bind to EGFR directly and suppress EGFR signaling, but they also bind other members of the ErbB family and are considered collective ErbB inhibitors. LRIG1, SOCS4 and SOCS5 half-life data suggests transcriptional and post-transcriptional control mechanisms that confine inhibitor expression to a window of a few hours [103], which could be extrapolated to Mig6 but direct studies on its half-life during EGFR activation are absent.

LRIG1 and SOCS4 have not been associated with metabolic disease, and SOCS5 has been loosely implicated (by association with the Jak/Stat pathway) with neuronal and cognitive function in diabetes [104]. Meanwhile, for Mig6, there is compelling evidence of metabolic effects in rodent models of diabetes [105-107].

Mitogen-inducible gene 6 (Mig6)

Mig6 is a catalytically inert 50 kDa protein containing an EGFR binding domain, a CRIB domain, a 14-3-3 protein binding motif, and a GRB2 binding motif [108]. Of note, the GRB2 motif contains SH2 and SH3 binding domains designed to bind Grb2, Src

kinase and phospholipase C γ (PLC-γ) (**Figure 1-4 A**). Mig6 transcription is rapidly induced by mitogens (such as EGF, HGF, and insulin) and stress stimuli (such as cytokines and hypoxia) [105].

Dimerization and activation of the EGFR induces Mig6 to bind all ErbB family members and inhibit tyrosine kinase activity. Mig6 is thought to function with two mechanisms to control EGFR signaling. First, by binding between the two EGFR kinase domains and preventing kinase activation [108] and second, by promoting protein scaffolding interactions that lead to internalization of the receptor [109, 110]. A simplified schematic in **Figure 1-4 B** illustrates Mig6 inhibitory mechanisms described above.

Mig6 has fairly ubiquitous expression patterns in adult human tissue, but is differentially expressed in embryonic tissues, with higher expression in the heart, fibroblasts, ovary, thyroid, pancreas, and liver (RNASeq data, GeneCards). Indeed, whole body Mig6 knock out mice have 50% embryonic lethality, and those that survive have abnormal lung development as measured by decreased ductal growth, density and branching [111]. Although there are few studies centered on Mig6 and development, the importance of EGFR signaling in development is well-established [92, 93, 97, 112], and substantiates the importance of Mig6 in development.

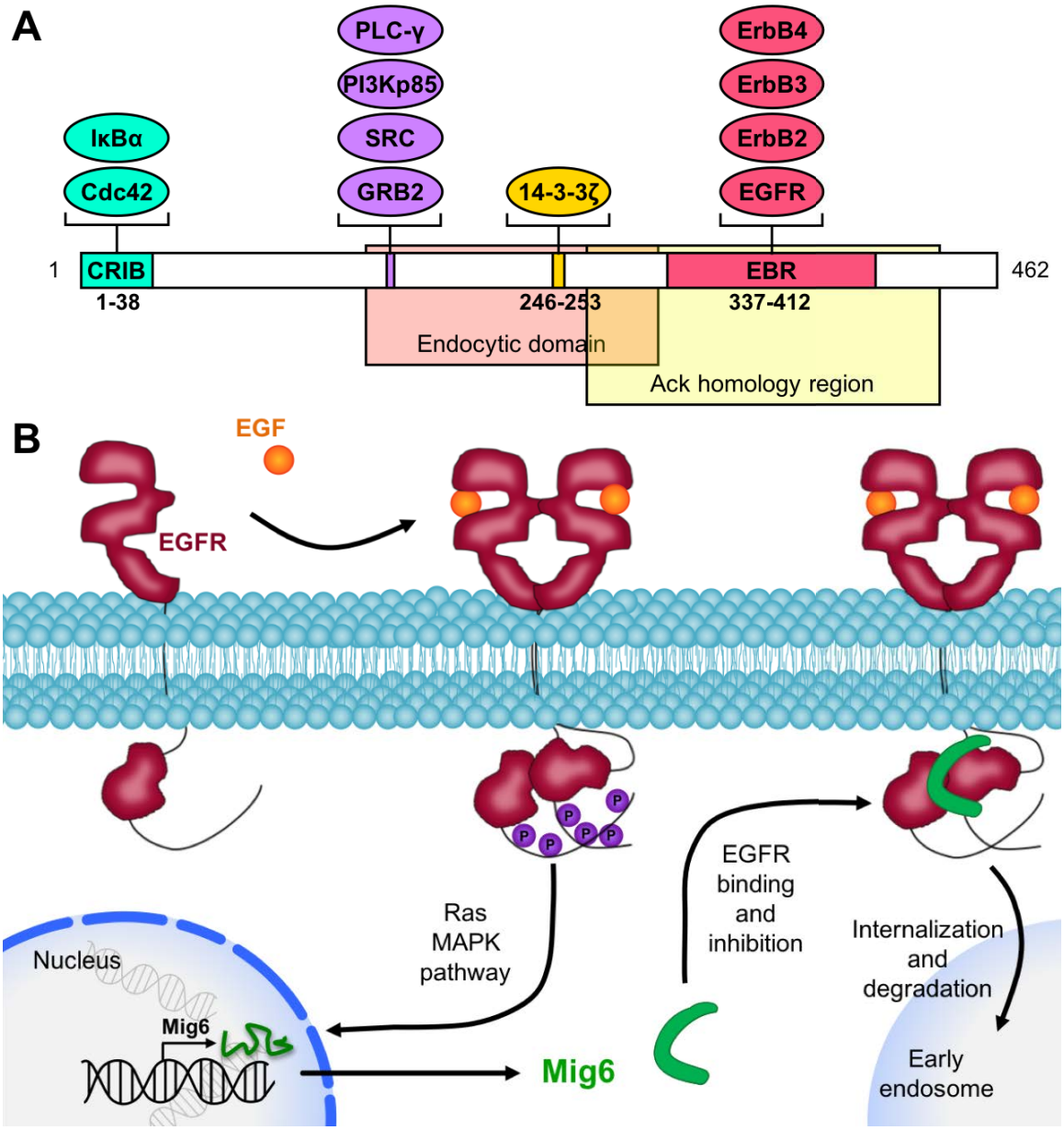


Figure 1-4. Mig6 structure and function as an inducible feedback inhibitor of EGFR. (**A**) Structural domains of Mig6 and its binding interactions *modified from [112]*. (**B**) Mig6 mechanisms of EGFR inhibition.

Activation and priming of Mig6

Mig6 is induced by ER stress, glucolipotoxicity, and pro-inflammatory cytokines [105-107]. The mechanism(s) through which cytokines induce Mig6 expression, like EGFR inactivation, are unclear. Mass spectrometry has recently uncovered phosphorylation of Mig6 on several residues, including Ser251, Ser256, Tyr394, Tyr395 [111-115], suggesting that Mig6 is subject to post-translational control. A study recently confirmed tyrosine phosphorylation of Mig6 and suggested an activated Src family kinase (SFK) was involved [113]. There are 9 members of the SFKs [116] listed in **Table 1-2**. Each of the SFKs share similar structural features including a Src homology domain. Functional redundancy among members of the SFKs makes identification of the specific role of each Src kinase difficult. The activation of SFKs by many diverse families of receptors induces cellular responses that affect growth control, survival and differentiation, cytoskeletal arrangements, secretion, channel function and other biological activities [117].

Src, a non-receptor tyrosine kinase, is one of the most studied members of the SFKs, and has been implicated in proliferation, survival, apoptosis and differentiation [118]. Src is activated by receptor tyrosine kinases, like EGFR, as well as other stimuli that are altered in diabetes pathogenesis, such as G-protein coupled receptors, TGF-β, and reactive oxygen species (ROS) [119-121]. In fact, EGF treatment induces a two- to threefold increase in Src catalytic activity [122]. Additionally, it was demonstrated that treatment with IL-1β and TNF-α increased Src activation in human brain (T98G) and muscle (HTSM) cells, respectively [123, 124].

Table 1-2. The nine Src family kinases

SFK	Expression [125]	Selected Functions [117, 126-146]
Src	Ubiquitous, two neuron-specific isoforms	Cell adhesion Focal adhesion dynamics Cell migration Integrin-induced MAPK activation Cell cycle progression Differentiation reviewed in [138]
Fyn	Ubiquitous, T cell-specific isoforms	Focal adhesion dynamics T cell development Cell cycle progression
Yes	Ubiquitous	Cell cycle progression
Lyn	Brain, B cells, myeloid cell; two alternatively spliced forms	Apoptosis promotion or prevention*
Hck	Myeloid cells, bone	Bone remodeling
Fgr	Myeloid cells, B cells, blood, bone, spleen	Apoptosis promotion or prevention*
Blk	B cells, lymph node, spleen, appendix	B cell receptor signaling, B cell development, stimulates insulin synthesis and secretion in response to glucose and enhances the expression of pancreatic β cell transcription factors [147]
Lck	T cells, NK cells, brain, thymus	T cell migration and maturation, Apoptosis+[117]
Frk	Epithelial cells	Cell cycle suppression [125]

*ambiguous function, +speculative function

Although it has traditionally been referred to as an adapter protein, phosphorylation ('priming') of Mig6 on tyrosine residues 394 and 395 suggests it is subject to its own regulatory mechanism. The interplay among Src activation, Mig6 activation, and EGFR inactivation, all mediated by pro-inflammatory cytokines and ROS, has yet to be investigated. Src kinase is a likely candidate for Mig6 regulation and warrants further study to fully understand mitogenic signaling and its potential in expansion of β cell mass.

1.5 β cell regeneration

Strategies to promote β cell regeneration during the progression to T1D can be used to restore functional β cell mass. During development, β cell neogenesis occurs to increase β cell mass in fetuses. Understanding of this process may be transferred to current work on stem cell-derived β-like cells for transplantation or drug development. Another area of focus is the immunological attacks and triggers and how it can be targeted to altogether prevent the loss of β cell mass. Lastly, there is significant work around restoring lost β cell mass from endogenous sources, which may include recycling developmental mechanisms to regenerate β cell mass after diabetes onset.

Clinical instances of human β cell regeneration

Pancreas sections obtained from autopsy in healthy and T1D patients reveal that there are β cells present in the majority of patients with longstanding T1D, and that β cell number does not correlate with duration of disease, which was the case even though the number of β cells undergoing apoptosis was doubled in T1D islets [148]. Presence of scattered single β cells and β cells within islets from T1D pancreas samples has also been reported [149, 150]. Moreover, in the unique case of an 89-year old patient who was recently diagnosed with T1D and received a pancreatectomy, cellular immunostaining not only confirmed that β cell apoptosis is an important mechanism in T1D-mediated β cell destruction, but also that β cell regenerative mechanisms are initiated in recently-onset T1D [151].

Endogenous sources of *in vivo* β cell regeneration

Current advances in islet replacement therapy have been shown to reverse diabetes in 88% of patients at 1 year and 71% at 2 years, but by 5 years post-

transplantation, only 10% of patients remain insulin independent [152, 153]. The surgery costs about $20,000 and pooled islets from several donors are transplanted into one patient with T1D. Therefore, cadaveric islet transplantation is not a suitable widespread treatment because the ratio of available donors to potential patients is too small and too costly to repeatedly treat the general population. In addition, chronic immunosuppression is required for success of the transplant, which can also produce unwarranted effects such as tumors and insomnia [154]. Instead, treatment for the general population is intensive insulin therapy. While insulin therapy has drastically improved outcomes for patients with T1D, compared to exacting biological islet cells, it is a crude means of controlling BG levels and often is accompanied by hypoglycemia. Strategies that mine endogenous pools of insulin-producing cells are increasingly attractive to replace β cells and attain physiological BG control [155]. Such strategies include β cell proliferation of existing endocrine cells, differentiation of embryonic stem cells (ESCs) to β cells, or transdifferentiation of exocrine stem/progenitor cells into β cells [156-159].

Proliferation of existing β cells is thought to be one endogenous source of β cell replenishment. Significant efforts went into identifying compounds that could stimulate β cell proliferation, resulting in identification of several compounds that are capable of stimulating β cell expansion in rodent islets [160-162]. Although it was found that newly-onset diabetes manifested in increased β cell death, there is only some evidence that the proliferative regeneration program, specifically, is present in the human pancreas [163].

Neogenesis is the formation of new islets cells from pancreatic progenitors (**Figure 1-3**) and is accepted as the method of initial β cell expansion during embryogenesis [157]. Whether these pancreatic progenitors retain their plasticity into

adulthood is a subject of some controversy, but several studies support that they do retain some degree of plasticity. For example, GLP-1 receptor agonists have been reported to induce β cell replication and β cell neogenesis from pancreatic progenitor cells in adult rodent models [164]. It was also demonstrated that acinar cells, which represent a major proportion of the developed pancreas, can transdifferentiate into insulin-producing cells [165]. ESCs and induced pluripotent stem cells represent the most widely investigated candidates for *in vitro* reprogramming because they can theoretically proliferate forever and have a high potential to differentiate [157].

The pancreatic ducts also possess a potential subgroup of cells that can give rise to insulin-producing cells *in vitro* [166, 167]. Much evidence exists supporting β cell regeneration from the duct epithelium [38, 168, 169]. For instance, in human and mouse cells, it was demonstrated that cells within the pancreatic ductal gland proliferate and commit to β-like cells under high glucose conditions [170]. Also, lineage tracing of EGF/gastrin-regenerated β cells found that the new β cells were budding from the duct during postnatal development [99]. This particular pool of progenitor cells represents a promising source of cells that can be made into β-like cells *in vitro* by targeted delivery of crucial transcription factors.

Zebrafish as a model for pancreas development and β cell regeneration

Zebrafish are a convenient model for pancreas development because they are easy to manipulate genetically, they are relatively inexpensive, embryos can be quickly collected in large quantities year-round, and they have β cell regeneration capabilities [171, 172]. Furthermore, of particular importance, the pancreatic developmental program

as well as actions of glucose regulating proteins (insulin, glucagon and others) are highly conserved between mammals and zebrafish [173-176].

As described previously, transcription factors determine cell specification in human pancreas development. As in mammals, zebrafish *pdx1* and *ptf1a* are necessary for regulating pancreatic progenitor differentiation, and its expression can be tracked in the developing pancreas tissues: from dorsal bud progenitors at 12 hpf to β cells at 24 hpf, and finally to the ventral pancreas after ventral bud specification, where they remain to maintain acinar cell identity [177]. *neurod1, arx, nkx2.2, pax6, mafa* are all conserved transcription factors as well. Curiously, whereas rodent *Ngn3* is required for β and α cell specification, zebrafish *ngn3* is not required for endocrine cell formation. *Ngn3* function appears to be replaced in zebrafish by *ascl1b*; *ascl1b,* together with *neurod1,* regulates differentiation of pancreas progenitor cells into endocrine cells [178]. Altogether, zebrafish have conserved endocrine cell development and represent a unique and relevant model of the mechanisms involved in pancreas development.

Zebrafish also have the capacity to regenerate β cells after chemically-induced islet ablation [172, 179]. Some of the differentiation mechanisms described above may be involved in the regenerative capacity of zebrafish. Specifically, **Figure 1-5** depicts duct progenitor cell differentiation into endocrine cells and their migration to the principal islet of zebrafish during early pancreas specification. A β cell ablation and regeneration model using transgenic lines expressing nitroreductase (NTR) in insulin-producing cells is a powerful tool to study β cell regeneration. The reduction of NTR and its products' reaction with metronidazole (MTZ) produces a cytotoxic environment in the NTR-expressing β cells causing both ROS generation and cross-linking of DNA strands that

induces β cell death [172, 180]. After a period of recovery, β cell regeneration can be precisely quantified and origins of those cells can be simultaneously studied by lineage tracing studies.

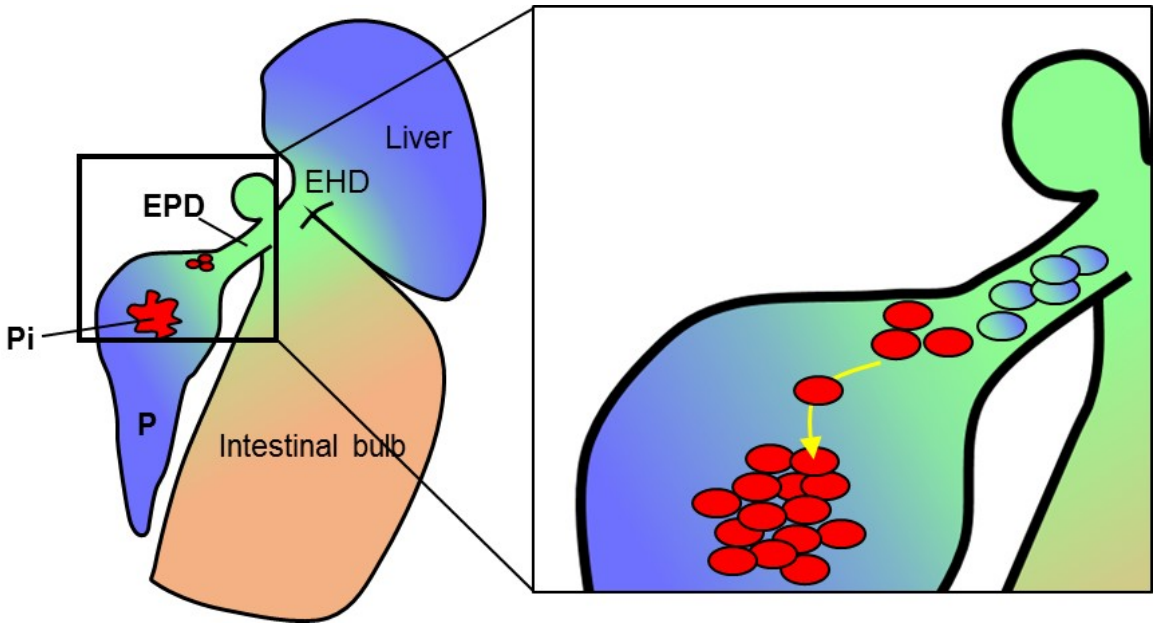


Figure 1-5. Schematic diagram of β cell regeneration and migration from progenitor cells in the zebrafish. EPD; pancreas = **P**, principal islet = **Pi**, extrapancreatic duct = **EPD**, extrahepatic duct = **EHD**. endocrine cells = **red**, progenitor cells = **blue**+**green**

To summarize, we perceived a gap in understanding of Mig6 regulation and control mechanisms. Mig6 has potential therapeutic applications to increase functional β cell mass via indirect EGFR manipulation. Given the extensive impact of EGFR signaling on developmental pathways and protection against diabetes, we suspected that Mig6 had an important function in pancreas development and in the progression to diabetes. Notably, cytokines 1) induce Mig6 expression and 2) stimulate β cell death and leads to loss of β cell mass during the progression to diabetes. We propose that cytokine-

induced Mig6 results in a loss of EGFR signaling which impedes β cell regeneration in the immune cell infiltrated islet.

1.6 Aims and Hypotheses

We aimed to investigate the role of Mig6 in development and identify the molecular events by which Mig6 affects β cell mass during the progression to diabetes. We used a zebrafish model of development to study Mig6 during pancreatogenesis and based on observed abnormalities in the ducts of Mig6 knock out mice [181], we hypothesized that loss of Mig6 increases EGFR activation in the duct, causing early differentiation of duct progenitor cells, thereby decreasing potential β cell regeneration. We investigated a proposed mechanism of Mig6 regulation by inhibiting Src kinase in the 832/13 cell line, hypothesizing that cytokines induce Mig6 expression via activation of an SFK. Finally, we reinforced these data by characterizing Mig6 pancreas-specific knock out mice and elucidating the mechanism of EGFR inactivation resulting from cytokine-induced Mig6. We hypothesized that cytokine-induced EGFR inactivation by Mig6 accelerates the loss of β cell mass by blocking endogenous regenerative mechanisms.

CHAPTER 2. DIFFERENTIAL REGULATION OF DUCTAL NETWORKS BY MIG6 DURING ZEBRAFISH DEVELOPMENT

2.1 Summary

Strategies to promote β cell neogenesis could be used to restore functional β cell mass in diabetes. We examined the role of *mig6* in the regulation of functional β cell mass. *mig6* is evolutionarily conserved and encodes a protein that inhibits the EGFR in a classic feedback mechanism, suggesting a potential role in cellular growth and development. Based on functional studies in mouse and human, we hypothesized that *mig6* is required in pancreatic ducts for pancreatogenesis and β cell function. We used morpholino knock down in zebrafish to define the role of mig6 in both exocrine pancreas and islet development. We established that *mig6* knock down (*mig6MO*) embryos have a disrupted pancreas morphogenesis, particularly, a truncated exocrine pancreas and fewer intra-pancreatic duct cells. Additionally, *mig6MO*-injected animals had fewer α and β cells than controls.

Our data led us to hypothesize that *mig6* may facilitate the expansion of the pancreas progenitor pool by blocking EGFR activation. We further investigated the role of *mig6* in islet regeneration. Neogenic β cells may arise from two sources in the developing zebrafish, which can be distinguished by the retention of a fluorescent mark made at the one-cell stage. β cells derived via transdifferentiation from other post-mitotic endocrine cells remain labeled, whereas those derived from duct-associated progenitors lose their label via proliferation. Together, our data demonstrate that *mig6* is essential for pancreas development and that it might be exploited as a switch between progenitor

differentiation and endocrine transdifferentiation to increase β cell mass in diabetic patients.

2.2 Introduction

Both T1D and T2D are characterized as the loss of the insulin-producing pancreatic β cells, demonstrated by a reduction in functional β cell mass. Functional β cell mass may be restored through methods such as α to β cell transdifferentiation, β cell proliferation, and β cell neogenesis [182]. To restore β cell mass from undifferentiated cell types, it is essential to understand what controls the fate of the pancreatic and duct progenitor cells. Human pancreatogenesis studies have revealed several of the crucial transcription factors directing β cell differentiation and neogenesis, including *MAFA*, *PDX1*, *PTF1A*, *PAX6*, *NGN3*, *FOXA2* and insulin. However, our understanding of the generation of β cells in human is limited due to the challenge of establishing primary cell lines of these progenitors yielding to long term cell culture and adaptation.

EGFR signaling has been intensively studied during both development and the progression to diabetes. EGFR knock out mouse embryos have a smaller pancreas [94] and EGFR is required for ductal growth, density and branching in mouse mammary glands [183]. As a feedback inhibitor of EGFR signaling, *mig6* is an obvious candidate for roles in pancreas and duct development. In fact, during development, *mig6* seems to be crucial for branching in the lung [184], an organ whose proper function requires an extensive ductal network. An extensive ductal network is also essential in the liver and pancreas. To this end, manipulating EGFR-mediated cell survival and proliferation in the

pancreas by targeting *mig6* may play a role in maintaining euglycemia and regenerating β cells.

Developmental studies utilize the diverse and widely applicable zebrafish model because of the short gestation period, whole mount visualization of genes and proteins, and easily manipulated gene expression. A potential source of regenerated β cells in humans and various animal models is the differentiation of ductal network endocrine progenitor cells [182, 185]. Numerous *in vivo* rodent studies have revealed evidence that pancreatic ducts are a source of progenitor cells [38]. Findings that lead to this understanding included partial pancreatic tissue regeneration from ductal cells [186], appearance of β cell clusters from ductal epithelium 3-days post 90% pancreatectomy [187], and the conversion of duct specific *Sox9*$^{+}$ cells into insulin-producing cells after the combined treatment of EGF and gastrin [188]. After β cell injury, embryonic zebrafish can restore β cells, and within 3 to 4 days recovery time, can regenerate β cells to restore normal islet function and much islet architecture [179, 189]. In the zebrafish, studies propose that a component to this regeneration utilizes the natural development process of islet formation, when late endocrine β cells forming from the ventral bud migrate within the pancreas to join the principal islet [185, 190].

2.3 Results

Mig6 is conserved across species

Genes and proteins of various animals may have various and nuanced functions within different organisms. We performed an amino acid sequence alignment comparing Mig6 among human, mouse, and zebrafish. NCBI Protein Blast was used to calculate

percent identity. Human and mouse Mig6 are 82% homologous, whereas human and zebrafish Mig6 are just 45% homologous. The function, structure, and mechanism of Mig6 as an EGFR inhibitor in human and mouse is described considerably [108, 109, 181], so to determine functional similarities to zebrafish *mig6,* we analyzed the EGFR kinase binding domain sequence separately. It became evident that the EGFR kinase binding domain is extraordinarily conserved across all three species. The homology of the isolated region is 100% between human and mouse, and 89% between human and zebrafish (**Figure 2-1 A-B**). A sequence alignment of the EGFR protein determined 88% identity between human and mouse, and 63% identity between human and zebrafish, with high sequence homology in the Mig6 binding site (*data not shown*). With these data, we concluded that the Mig6-EGFR relationship and function in zebrafish was likely to be highly comparable to, if not identical to its relationship and function in human and mouse.

***mig6* is expressed in zebrafish**

To date, no studies have examined *mig6* in zebrafish development. So, we report here the first expression data in this animal model. Temporal *mig6* expression from whole-body zebrafish embryos revealed that *mig6* was expressed at 2, 4, and 7 dpf, and of those times, it was most highly expressed at 4 dpf (**Figure 2-2**). Data from *in situ* hybridization of *mig6* pinpoint both its temporal and spatial expression during organogenesis and pancreatogenesis. Expression of the genes *insulin*, *fabp10*, and *mig6*, as well as the sense control probe for *mig6* was used to determine localized expression in the endoderm. By 3 dpf, *mig6* was strongly expressed in the heart. At 3 dpf, *mig6* expression was observed in the liver and *mig6* expression gradually increased in the liver

at 4 dpf and at 7 dpf. *Mig6* expression was most robust at 4 and 7 dpf where it was clearly expressed in the heart, liver, and pancreas (**Figure 2-3**). Further analysis of the endoderm by *in situ* hybridization of the genes *sox9b* and *mig6* revealed more specific expression patterns in the pancreas (**Figure 2-4 A-D**), but it appeared *mig6* was expressed in a similar pattern to *sox9b* in the pancreas (**Figure 2-4 E-L**).

***mig6MO* causes severe alterations in development**

To block production of Mig6 protein, we designed an ATG morpholino (*mig6MO*) targeting zebrafish *mig6* at exon 2 and a splice morpholino (*mig6MO2*) targeting the junction of exon 3 and intron 3 (**Figure 2-5 A**). Injection of the 'standard doses' of either 2 or 4 ng of *mig6MO* was lethal to embryos at an early stage (*data not shown*). This observation is consistent with our assertion that *mig6* is essential for development. We determined a dose range of 0.25 to 0.50 ng produces viable embryos for further study. Injection of *mig6MO* disturbed normal development in the zebrafish. Upon gross examination, *mig6MO*-injected embryos appeared stunted in growth and exhibited pericardial sac edema and an enlarged hindbrain ventricle (**Figure 2-5 B**) Insulin staining in the pancreas of the morpholino-injected embryos indicated that there was an altered pancreas phenotype in the *mig6MO*-injected embryos. We classified the various levels of morphological change into three categories: Normal, Class I, and Class II. Normal categorization was defined as a pancreas with tapered tail in combination with centered islet position (**Figure 2-5 C**). With this as a reference point, embryos with an uncentered islet (**Figure 2-5 D**) were classified as Class I, whereas embryos with the islet or partial islet protruding from the exocrine tissue (**Figure 2-5 E**) were classified as Class II. The separation of the islet and the exocrine tissue suggests that fusion of the dorsal

and ventral buds is impaired. The incidence of Class I and Class II embryos increased with increasing morpholino dose (**Figure 2-5 F**).

***mig6MO*-injected embryos have a truncated exocrine pancreas and an elongated extrapancreatic duct**

During further assessment of the physical changes between control and injected embryos, we discovered that *mig6MO*-injected embryos had truncated pancreata as measured by the length of the exocrine tissue (**Figure 2-6 A-C**) through the medial line of the pancreas from pancreas head to tip of the pancreas tail. Initially, we hypothesized that knocking down *mig6* would increase EGFR activity and result in growth of the pancreas. Interestingly and contrary to our expectations, the pancreas length decreased with increasing morpholino dose (**Figure 2-6 D**).

Because *mig6* is important to branching organs in the mouse, we were particularly interested in the development in the ducts. We stained control and *mig6MO*-injected embryos with gut secretory cell epitopes (2F11) to denote the ducts (**Figure 2-7 A-C**). In the *mig6MO*-injected embryos, the duct between the gall bladder and the pancreas, called the extrapancreatic duct (EPD), had a significant increase in length and area versus the control embryos (**Figure 2-7 D-E**).

***mig6* knock down results in fewer α and β cells**

The EPD gives rise to late endocrine cells that migrate and contribute to the expansion of the principal islet (**Figure 1-5**) [191-194], so we suspected that endocrine cell fate had also been altered with the change in EPD size. To analyze the impact of *mig6* knock down to the pancreatic endocrine cells, we stained control and *mig6MO*-injected embryos with insulin and glucagon and counted the number of α and β cells

(**Figure 2-8 A**). Whereas control embryos had an average of 28 β cells, *mig6MO*-injected embryos had 20 β cells on average (**Figure 2-8 B**). In addition, control embryos had 34 α cells, but *mig6MO*-injected embryos had just 21 α cells (**Figure 2-8 C**).

***mig6MO*-injected embryos develop more β cells from the ventral bud**

Cells that develop from the ventral and dorsal buds were tracked by a label retaining cell (LRC) assay. The dorsal pancreas forms within 40 hpf and includes formation of the principal islet, whereas the ventral pancreas forms by 76 hpf and includes mostly acinar and exocrine pancreas cells [195]. The timing of this development, is consistent with cells in the ventral pancreas dividing at a higher rate than those in the dorsal pancreas, allowing the identification of cells that are dorsal bud- or ventral bud-derived. To this end, embryos were injected at the single cell stage with a fluorescent dye (H2B-RFP). Increased dilution of the dye is inversely related to intensity of fluorescence, so that more frequently dividing cells have more dilute dye and less intense red fluorescence. To evaluate derivations of β and α cells, control and *mig6MO*-injected embryos were stained at 4 dpf with insulin and glucagon. Insulin-positive cells with H2B dye present were designated LRC+ β cells (dorsal bud-derived); insulin-positive cells without H2B dye were designated LRC– β cells (ventral bud-derived). Glucagon-positive cells with and without dye were categorized similarly. In the *mig6MO*-injected embryos, significantly more β cells were derived from the ventral bud (**Figure 2-9 A**), with the dorsal bud trending to produce fewer β cells. Interestingly, fewer α cells were derived from the dorsal bud (**Figure 2-9 B**).

A

```
Zebrafish  MRTDCSWSMSTAGLTAQEICLPTQSLFLRGSHWHSMAGAKPSWKC-NDLRNLYLSIDSST  59
Mouse      --------MSTAGVAAQDIRVPLKTGFLHNGQALGNM-K-SCWGSHSEFENNFLNIDPIT  50
Human      --------MSIAGVAAQEIRVPLKTGFLHNGRAMGNM-RKTYWSSRSEFKNNFLNIDPIT  51

Zebrafish  -DYNIES--QKKVLPTSYSAVEKQRSHNNSTNSTG---AQPLPPKKPRPSQLTLHTEPYT  113
Mouse      MAYNLNSPAQEHLTTVGCAARSAPG-SGHFFAECGPSPRSSLPPLVI--------SPSES  101
Human      MAYSLNSSAQERLIPLGHASKSAPM-NGHCFAENGPSQKSSLPPLLI--------PPSEN  102

Zebrafish  SNPAKDDQVVPCFERLTVSDRIS--PPQT--------------PSRATKPLPPIPGSAEL  157
Mouse      SGQREEDQVMCGFKKLSVNGVCTSTPPLTPIKSCPSPFPCAALCDRGSRPLPPLPISEDL  161
Human      LGPHEEDQVVCGFKKLTVNGVCASTPPLTPIKNSPSLFPCAPLCERGSRPLPPLPISEAL  162

Zebrafish  SPDQAMDSEVEFFNGDENHCLVSESCSKNSPFRYGMPSRRSFRGCGQINYAYFEGPTSQQ  217
Mouse      CVDE-ADSEVELLTTSSDTDLLLE-DSAPSDFKYDAPGRRSFRGCGQINYAYFDSPTVSV  219
Human      SLDD-TDCEVEFLT-SSDTDFLLE-DSTLSDFKYDVPGRRSFRGCGQINYAYFDTPAVSA  219

Zebrafish  KQQQQQQKARQERENQQREQELRDQQQNCIRQQERTQRKLRRSHSGPAGCFNKTASFRYS  277
Mouse      ADLSCASDQNR-------------VVPDPNPPPPQSHRRLRRSHSGPAGSFNKPAIRISS  266
Human      ADLSYVSDQNG-------------GVPDPNPPPPQTHRRLRRSHSGPAGSFNKPAIRISN  266

Zebrafish  -SHH--RYTHNLDKPEVPPRVPIPPRPIKTGDNRRWSAEVSSGANSDDDRPPKVPPREPL  334
Mouse      -CTHRASPSSDEDKPEVPPRVPIPPRPAKP-DYRRWSAEVTSNTYSDEDRPPKVPPREPL  324
Human      CCIHRASPNSDEDKPEVPPRVPIPPRPVKP-DYRRWSAEVTSSTYSDEDRPPKVPPREPL  325

Zebrafish  SAC-PRTPSPKSLPIYYNGIMPPTQSFAPDPKYVSR-GLQRQNSEG----SPCILPVMEN  388
Mouse      SRSNSRTPSPKSLPSYLNGVMPPTQSFAPDPKYVSSKALQRQSSEGSANKVPCILPIIEN  384
Human      SPSNSRTPSPKSLPSYLNGVMPPTQSFAPDPKYVSSKALQRQNSEGSASKVPCILPIIEN  385

Zebrafish  GMKASNTHYFLLPQRPSYLDKHEKYLTDSTASRSTSASDSSSDWDCQSKR-----KTTHQ  443
Mouse      GKKVSSTHYYLLPERPPYLDKYEKYFKEAEETNPSTQIQ-PLPAACGMASATEKLASRMK  443
Human      GKKVSSTHYYLLPERPPYLDKYEKFFREAEETNGGAQIQ-PLPADCGISSATEKPDSKTK  444

Zebrafish  IDLV--------------                                            447
Mouse      IDMGSHGKRKHLSYVVSP                                            461
Human      MDLGGHVKRKHLSYVVSP                                            462
```

B

Figure 2-1. The EGFR kinase binding domain of Mig6 is conserved across species. **(A)** Amino acid sequence alignment of zebrafish, mouse, and human Mig6 protein by CLUSTAL Omega multiple sequence alignment software. (**B**) The EGFR kinase binding domain of Mig6 was isolated for sequence conservation and percent identity (% ID).

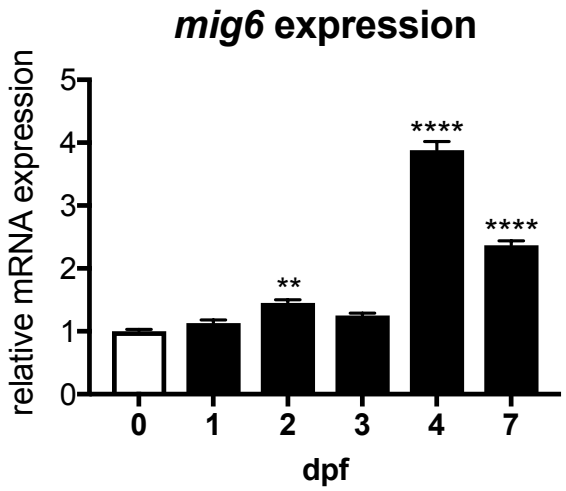


Figure 2-2. Dynamic expression of *mig6* during pancreas development. *mig6* expression was determined by qRT-PCR and normalized to the housekeeping gene *β-actin*. Data are presented as means ± SEM.

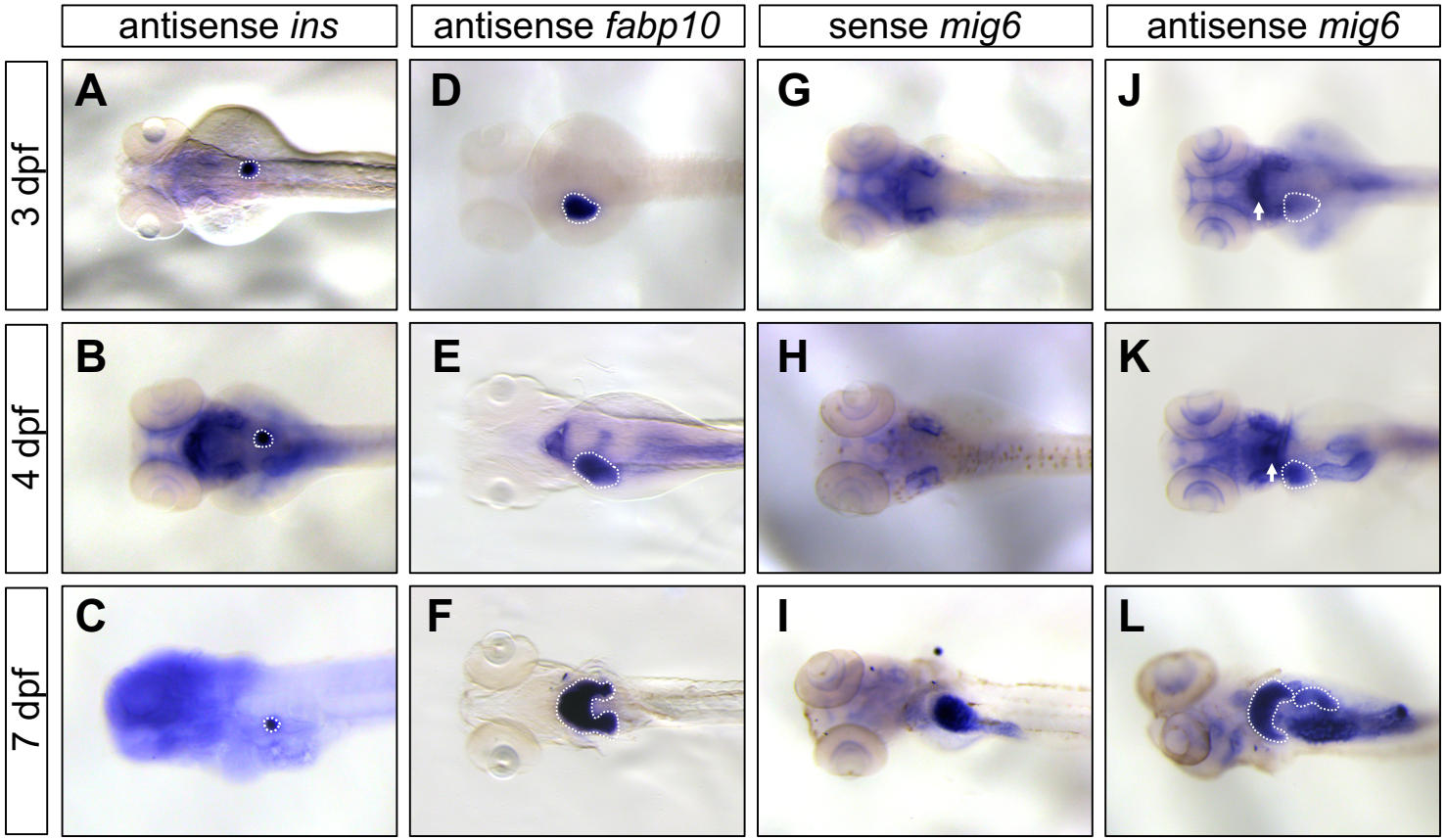


Figure 2-3. *mig6* expression patterns in the heart, liver, and pancreas at various developmental stages. **(A-L)** Images of *in situ* hybridization document the temporal and spatial distribution of the genes *insulin* (A-C), *fabp10* (D-F), and *mig6* (G-L) in zebrafish embryos 3, 4, and 7 days post fertilization (dpf).

sox9b antisense
A
B
mig6 antisense
C
D

ins antisense
sox9b antisense
fabp10 antisense
mig6 antisense
ISH, Tg(Nkx2.2:GFP), TO-PRO
E
F
G
H
BMP
I
J
K
L

Figure 2-4. *mig6* is expressed in the pancreatic ducts. **(A-D)** Magnified images of *in situ* hybridization identify the localized temporal and spatial distribution of the genes *sox9b* (A-B) and *mig6* (C-D) in zebrafish embryos 3 dpf (**E-H**) Fluorescent *in situ* hybridization in *Tg(Nkx2.2:GFP)* 4 dpf embryos identified gene expression relative to the pancreatic ducts: *insulin* in the islet, *sox9b* in the pancreas, *fabp10* in the liver, and expression of *mig6*. Vector Red was used to visualize expression. **(I-L**) Embryos from (E-H) were developed with BM Purple to confirm alkaline phosphatase activity.

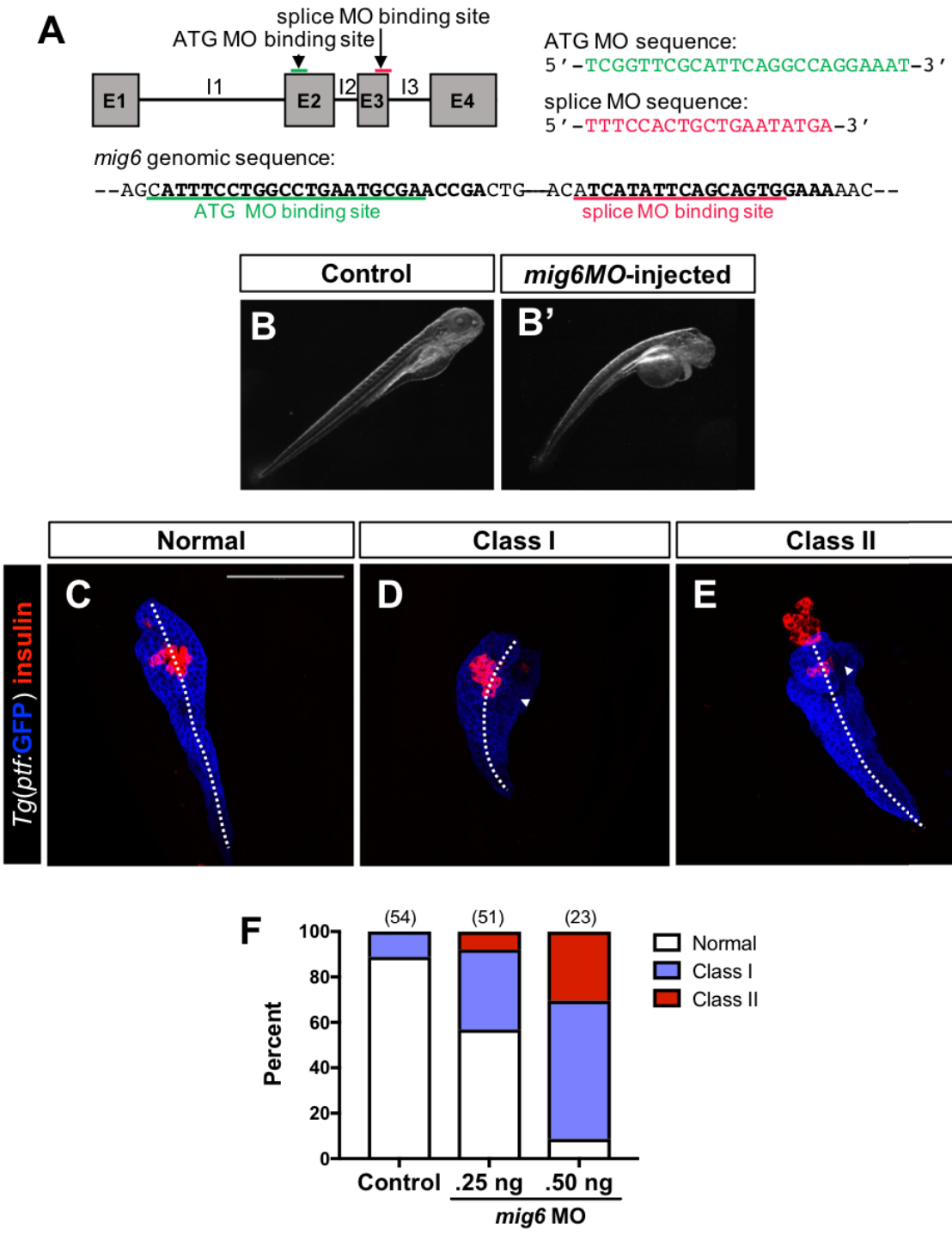

A
splice MO binding site
ATG MO binding site
E1
I1
E2
I2
E3
I3
E4
ATG MO sequence:
5′-TCGGTTCGCATTCAGGCCAGGAAAT-3′
splice MO sequence:
5′-TTTCCACTGCTGAATATGA-3′
mig6 genomic sequence:
--AGCATTTCCTGGCCTGAATGCGAACCGACTG—ACATCATATTCAGCAGTGGAAAAAC--
ATG MO binding site
splice MO binding site
Control
mig6MO-injected
B
B'
Normal
Class I
Class II
Tg(ptf:GFP) insulin
C
D
E
F
(54)
(51)
(23)
Percent
100
80
60
40
20
0
Control
.25 ng
.50 ng
mig6 MO
Normal
Class I
Class II

Figure 2-5. *mig6* morpholino causes morphological changes in the exocrine pancreas. (**A**) Representative diagram of the *mig6* gene with ATG and splice morpholino sequences and binding sites. (**B**) Representative images of control and *mig6MO*-injected embryos at 4 dpf. The phenotypes observed were classified into groups by severity of deformity: Normal, Class I, and Class II. (**C-E**) Representative images of each classification demonstrate the distinct characteristics by which they were classified: Normal fish have a centered islet position within a normal exocrine pancreas. Embryos with an uncentered islet were classified a Class I, whereas fish with islets or β cells located outside the exocrine tissue were classified as Class II. *Scale bar = 100μm*. (**F**) Changes in phenotypic class with morpholino injection were quantified into percentages; *n=23-54.*

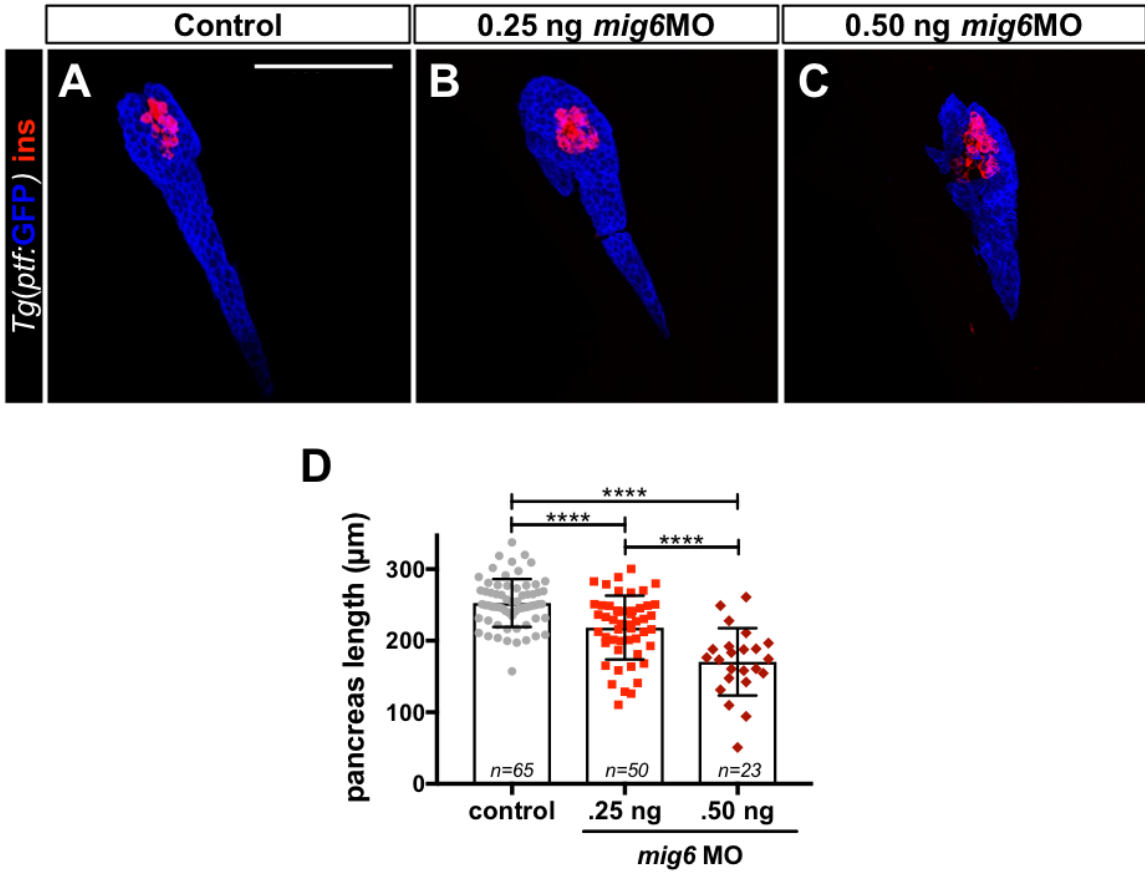


Figure 2-6. *mig6MO*-injected embryos have truncated exocrine pancreas. *mig6* morpholino was injected in two doses (0.25 and 0.50 ng) to zebrafish with the *ptf1a*:GFP transgene, and embryos were fixed at 4 dpf for 18-20h and stained for GFP and insulin. (**A-C**) Representative images of each dosage demonstrates the change in pancreas length. *Scale bar = 100µm.* (**D**) Exocrine pancreas length was measured by ImageJ analysis software with a segmented line between the furthest points of the exocrine tissue; *n=23-65; ****, p<0.0001, One-way ANOVA.*

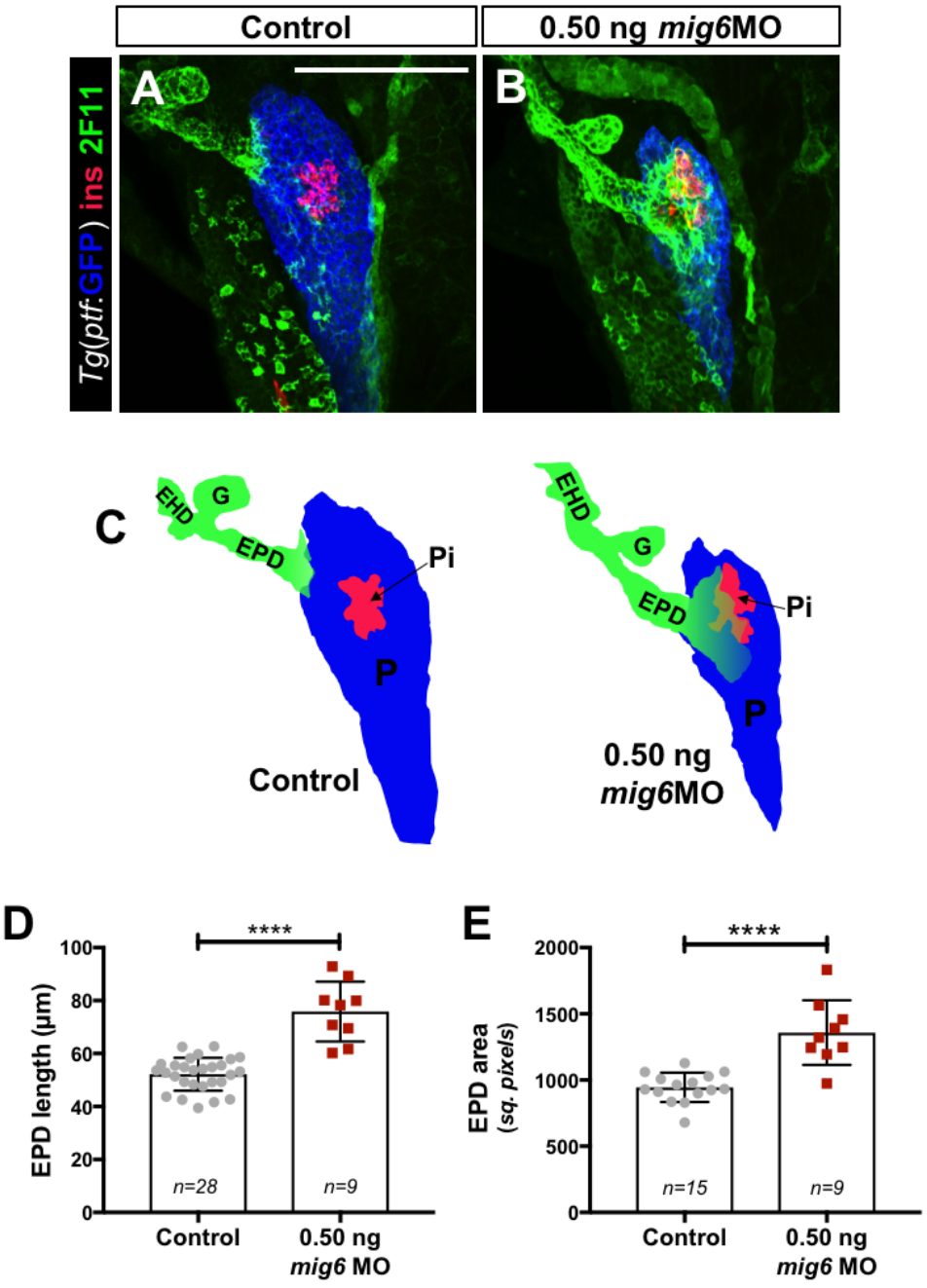

Control
0.50 ng mig6MO
Tg(ptf:GFP) ins 2F11
A
B
C
EHD
G
EPD
Pi
P
Control
0.50 ng
mig6MO
D
EPD length (µm)
****
n=28
n=9
Control
0.50 ng
mig6 MO
E
EPD area
(sq. pixels)
****
n=15
n=9
Control
0.50 ng
mig6 MO

Figure 2-7. Knock down of *mig6* results in an elongated extrapancreatic duct. (**A-B**) Representative images of the exocrine tissue marked by *Tg*(*ptf1a*:**GFP**), β cells marked by insulin (**ins**), and duct cells marked with **2F11**. *Scale bar = 100µm.* (**C**) Graphic representation of images in panels A and B, labeling relevant aspects of pancreas anatomy: pancreas (P), principal islet (Pi), extrahepatic duct (EHD), extrapancreatic duct (EPD), and gall bladder (G) in zebrafish. (**E**) The length and (**F**) area of the EPD, was measured using ImageJ software; ****, *p<0.0001, Student's t-test.*

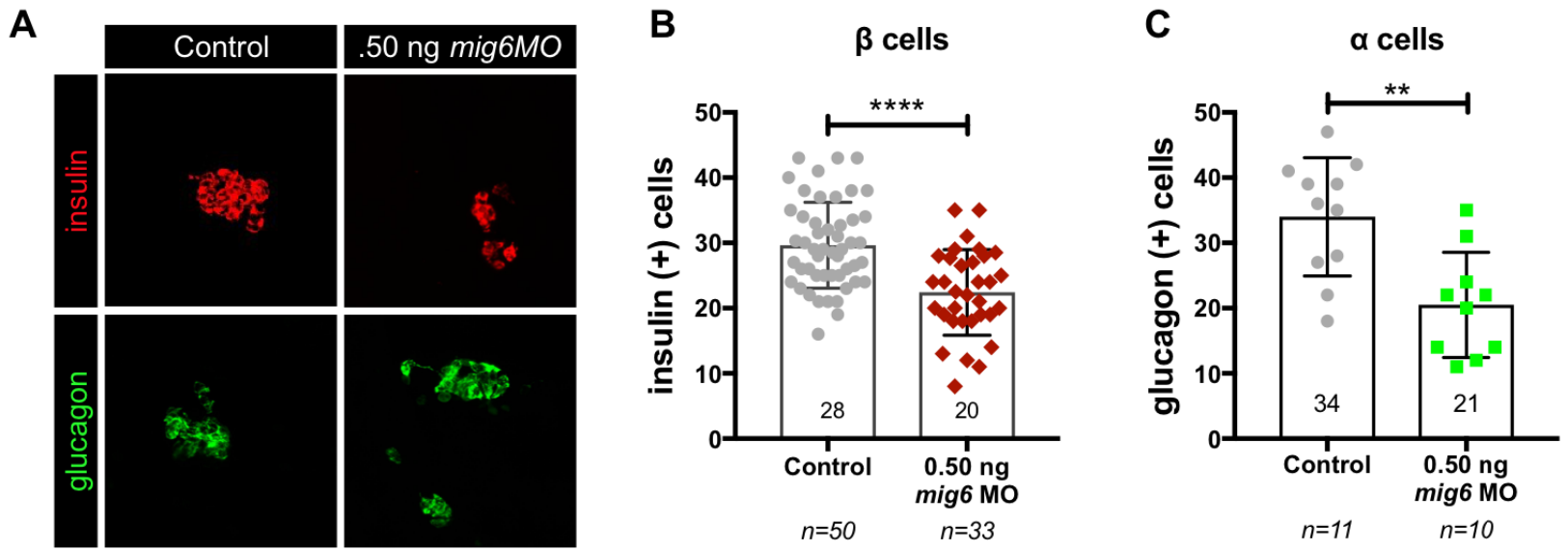


Figure 2-8. *mig6MO*-injected embryos have fewer β cells and fewer α cells. 0.50 ng *mig6* morpholino was administered to single cell embryos. Embryos were fixed at 4 dpf for 18-20 h and stained for (**A**) insulin (red), glucagon (green), and TO-PRO (not shown). Using ImageJ software, (**B**) numbers of β cells and (**C**) α cells were quantified; **, *p<0.01;* ****, *p<0.0001 Student's t-test.*

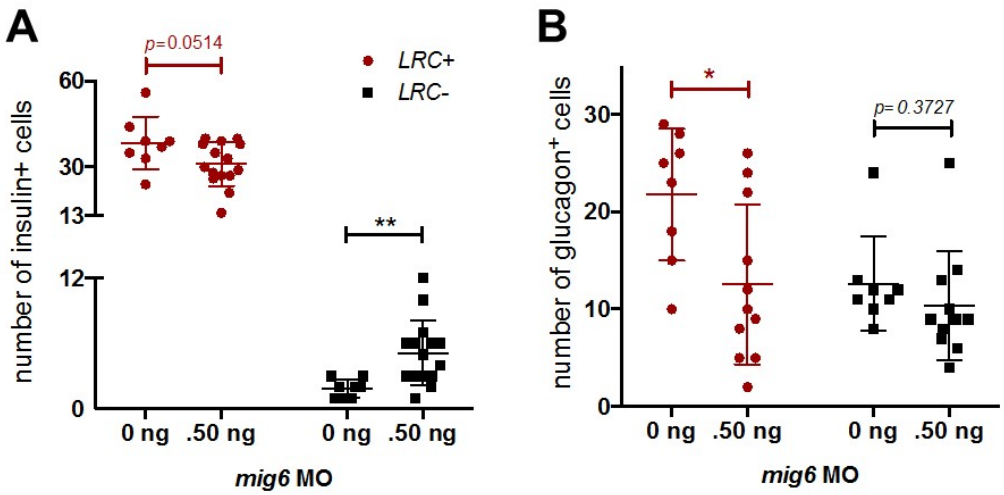


Figure 2-9. More β cells are derived from the ventral bud in *mig6MO*-injected embryos. Using a label retaining cell (LRC) assay, we separated cells based on frequency of division by monitoring dilution of a dye, H2B-RFP. We term cells in the dorsal bud-derived pancreas as LRC+ cells and those in the ventral bud-derived pancreas as LRC– cells. We counted the number of (**A**) insulin-positive LRC^{+} and LRC^{-} cells and the number of (**B**) glucagon-positive LRC^{+} and LRC^{-} cells in the control and *mig6* morpholino-injected embryos at 4 dpf; *, $p<0.05$,; **, $p<0.01$ *Student's t-test.*

2.4 Preliminary Results

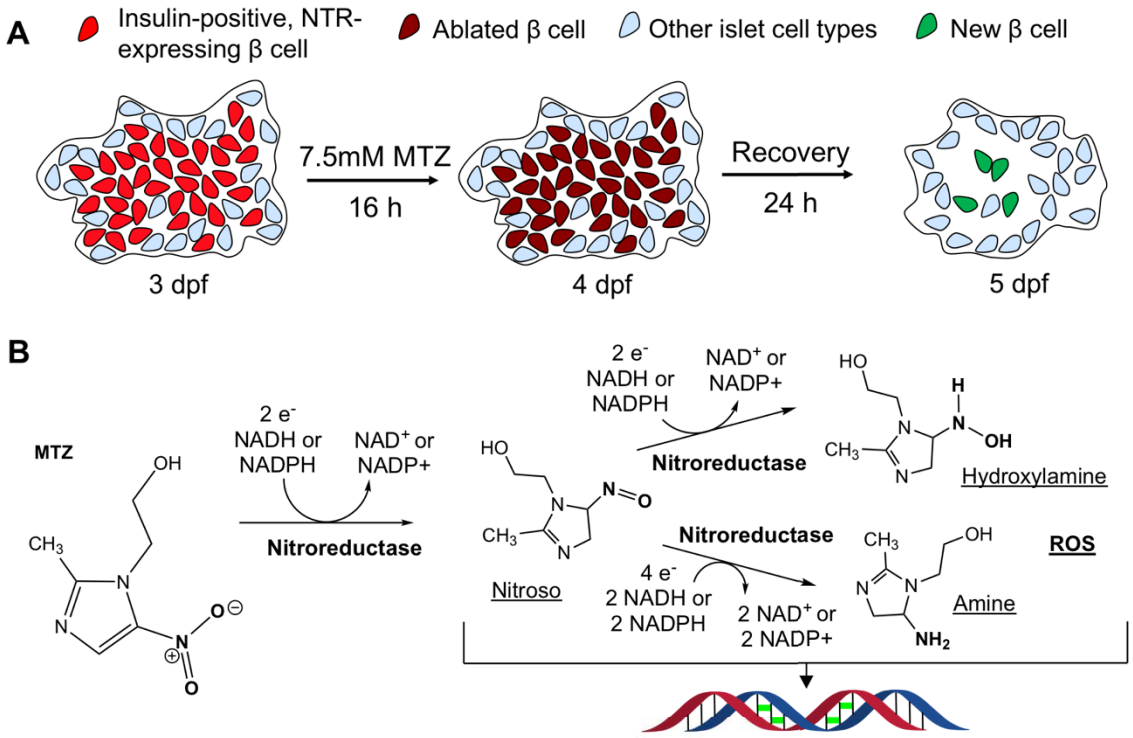
β cell regeneration in *mig6MO*-injected embryos

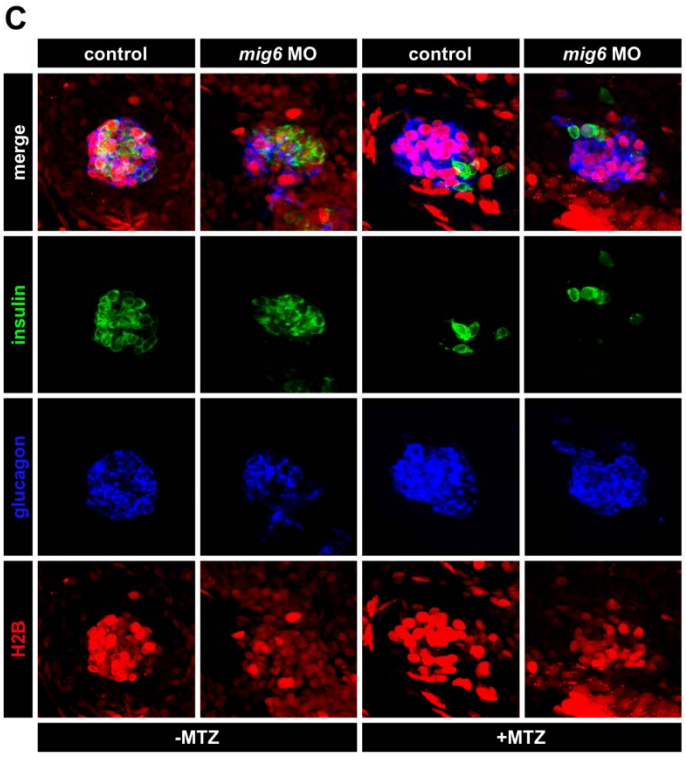
Previous studies in mice have uncovered that heterozygous whole-body knock out of Mig6 protects against β cell destruction and preserves β cell mass [105]. We hypothesized that *mig6MO*-injected embryos also have an improved response to stress stimuli. Utilizing the natural ability of zebrafish to regenerate their β cell mass, we treated control and *mig6MO*-injected *Tg(ins:CFP-NTR)* embryos with metronidazole (MTZ) to ablate β cells (**Figure 2-10 A**) by a proposed mechanism that generates ROS and cytotoxins that induce DNA damage (**Figure 2-10 B**). Embryos were allowed to recover for one day, then the regenerated β cells were quantified. There was no change in β cell regenerative capacity between control and *mig6MO*-injected embryos at 24 hours post ablation (hpa) (**Figure 2-10 D**). To determine the source of the regenerated β cells, the previously described LRC assay was used to separate dorsal bud-derived and ventral bud-derived cells. There was no difference in the numbers of β cells that were ventral bud-derived or dorsal bud-derived from control to *mig6MO*-injected embryos, although there was a difference from 0.25 ng to 0.50 ng *mig6MO* in β cells regenerating from the dorsal bud (**Figure 2-10 E**).

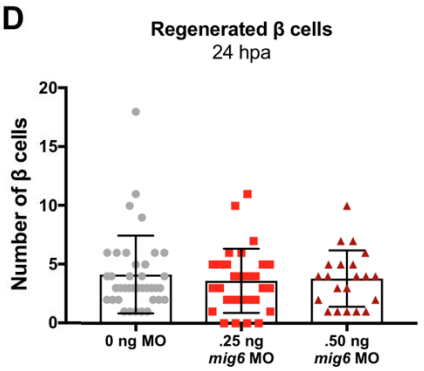
Generation of *mig6* mutants using CRISPR/Cas9

We used CRISPR/Cas9 to create *mig6* mutants and validate our *mig6MO* phenotype. To generate the CRISPR mutant, we designed a guide RNA (gRNA) target to exon 2 of the *mig6* transcript. Injection of *mig6* gRNA and Cas9 protein resulted in a 5-bp deletion in exon 2 (**Figure 2-11 A**). This 5-bp deletion was predicted to lead to a frameshift mutation and a premature stop codon (**Figure 2-11 B**). *Tg(ptf1a:GFP)*

embryos injected with gRNA were visually screened to verify a similar pancreas phenotype by truncated pancreas. Those embryos were allowed to grow into adults and were genotyped by fin clip. Not all visually identified embryos had consistently mutated sequences. For example, “putants” in **Figure 2-11 A** were all visually categorized as mutants based on pancreas phenotype, yet only two of the four had the 5-bp deletion. F1 generation zebrafish heterozygous for the mutation (confirmed by genotype) were crossed and the F2 generation offspring genotyped. Of the 18 random offspring taken for genotyping, 9 were wild-type (+/+) and 9 were heterozygous (+/-) for the mutation, but there were no homozygous mutants (**Figure 2-11 C**). Generation of a *mig6* mutant line is yet to be confirmed.


A
Insulin-positive, NTR-expressing β cell
Ablated β cell
Other islet cell types
New β cell
7.5mM MTZ
16 h
Recovery
24 h
3 dpf
4 dpf
5 dpf
B
MTZ
2 e-
NADH or NADPH
NAD+ or NADP+
Nitroreductase
Nitroso
Hydroxylamine
Amine
ROS
4 e-
2 NADH or 2 NADPH
2 NAD+ or 2 NADP+


C
control
mig6 MO
control
mig6 MO
merge
insulin
glucagon
H2B
-MTZ
+MTZ


D
Regenerated β cells
24 hpa
Number of β cells
0 ng MO
.25 ng mig6 MO
.50 ng mig6 MO

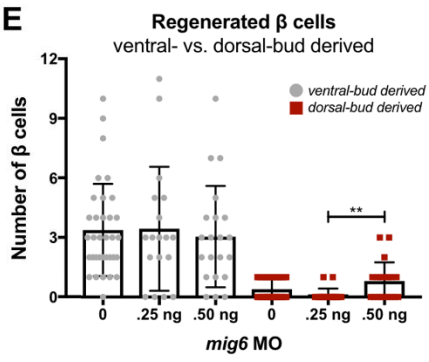

E
Regenerated β cells
ventral- vs. dorsal-bud derived
ventral-bud derived
dorsal-bud derived
Number of β cells
**
mig6 MO

Figure 2-10. There is no change in β cell regeneration between control and *mig6MO*-injected embryos at 24 hpa**.** (**A**) Schematic of experimental design of targeted and specific β cell ablation using the transgenic line *Tg(ins:CFP-NTR)* expressing nitroreductase (NTR) in the β cells. (**B**) The reduction of NTR and its products' reaction with metronidazole (MTZ) produces a cytotoxic environment in the NTR-expressing β cells, causing cross-linking of DNA strands (green) and inducing β cell death. (**C**) Representative images of islets from control and *mig6MO*-injected embryos treated with or without MTZ. Regenerating β cells were identified by antibody staining for insulin. (**D**) Total numbers of regenerating β cells (*n=21-38*) and (**E**) regenerating β cells separated into dorsal bud- and ventral bud-derived cells by LRC assay (*n=18-34*) were counted with ImageJ software. **, *p<0.01, One-way ANOVA.*

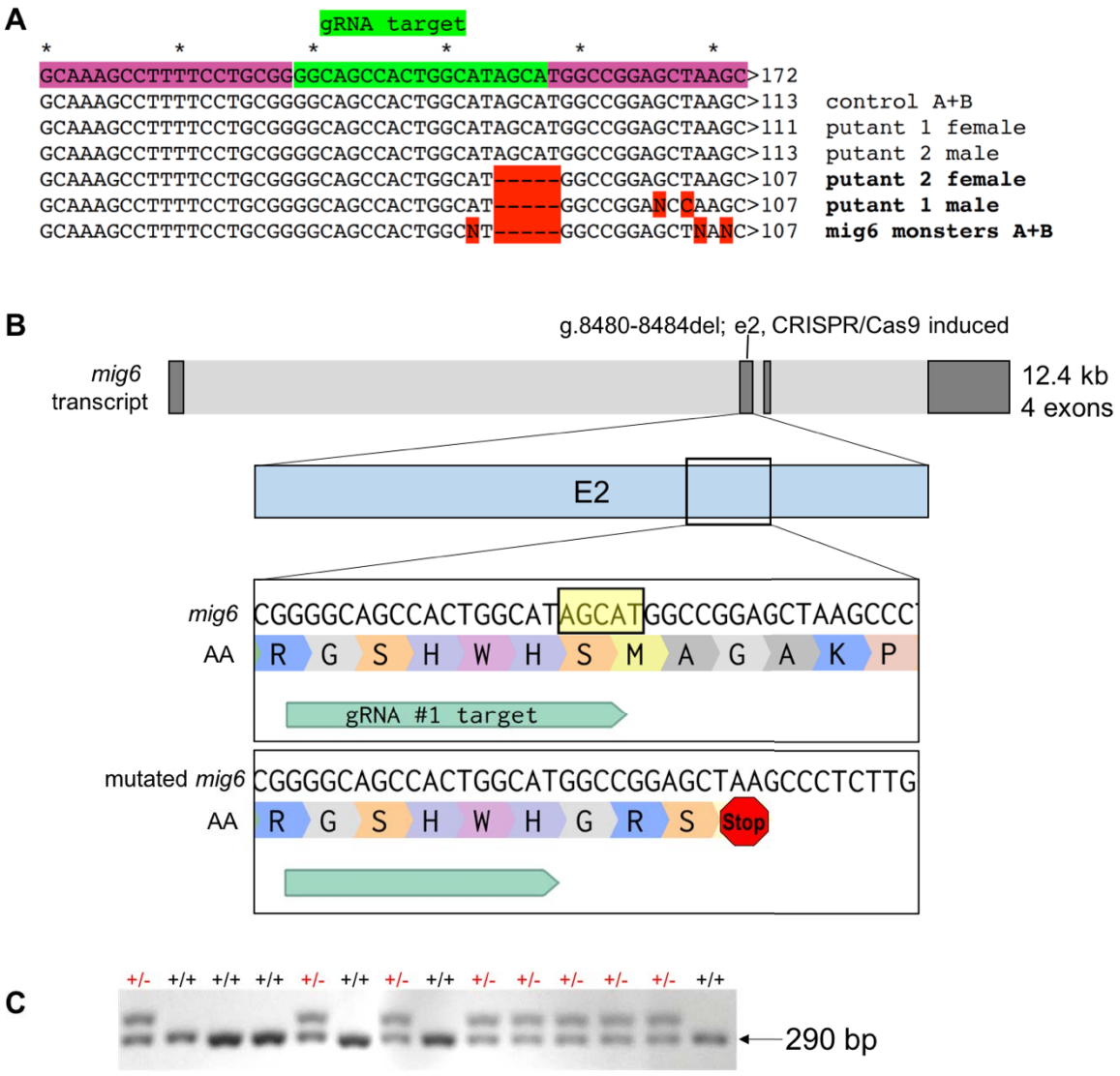


Figure 2-11. Generation of *mig6* mutants using CRISPR/Cas9**.** (**A**) Preliminarily, of 2 mutant embryos and pooled 'monster' embryos, all embryos had a 5-bp deletion in exon 2. (**B**) A 5-bp deletion in exon 2 (highlighted box) is predicted to lead to a frame shift mutation and premature termination codon. (**C**) F_1 generation mutants were crossed and offspring genotyped to confirm presence of germline mutation. Of the 18 random offspring screened, 9 were heterozygous (+/-) for the mutation, 9 were wild-type (+/+).

2.5 Discussion

Pancreatic growth and development involve several stages, utilizing pancreatic progenitor cell populations to expand insulin-producing cells by β cell proliferation, transdifferentiation, and neogenesis. These progenitor cells represent a population of cells that can be targeted to endogenously increase β cell mass. We investigated the role of a novel zebrafish gene, *mig6*, in the development of the pancreas and the differentiation of progenitor cells.

Alignment of amino acids of the human, mouse, and zebrafish Mig6 supports that the EGFR inhibitory function of *mig6* in zebrafish is conserved. We demonstrate that *mig6* is expressed in several different organs of the zebrafish, and although we focused on the pancreas and structures directly associated with the pancreas, we recognize that *mig6* may have supplementary roles in the heart and liver that contribute to the pancreatic phenotype described here. It is plausible that the *mig6MO*-injected liver might exhibit a change in bile duct morphology or change in liver size. Similarly, we must draw attention to the pronounced expression of *mig6* in the heart during development, and that the pancreas truncation in the *mig6MO*-injected embryos may be an effect of disrupted circulation-dependent pancreas growth. Circulation-dependent growth requires a healthy vascular network and has been proven necessary for liver development [196, 197]. Regardless, it is clear that the EPD augmentation is a primary result of a loss of *mig6* in the EPD. Cell growth in the EPD stimulated local duct progenitor cells to prematurely differentiate, as evidenced by the restructured proportions of exocrine tissue, EPD, β cells, and α cells.

We propose that *mig6* is a switch for progenitor cell differentiation. As depicted in **Figure 2-12**, in the absence of *mig6*, duct progenitor cells, which would normally give rise to α, β, and duct cells (among others), prematurely differentiate into terminally programmed cells. This explanation accounts for the observed enlarged EPD, truncated exocrine pancreas, and fewer β and α cells. In addition, studies in our lab have implicated *mig6* in Notch signaling, demonstrated by protein-protein interactions between Mig6 and the Notch signaling regulators, Numb and NumbL (unpublished data) [198].

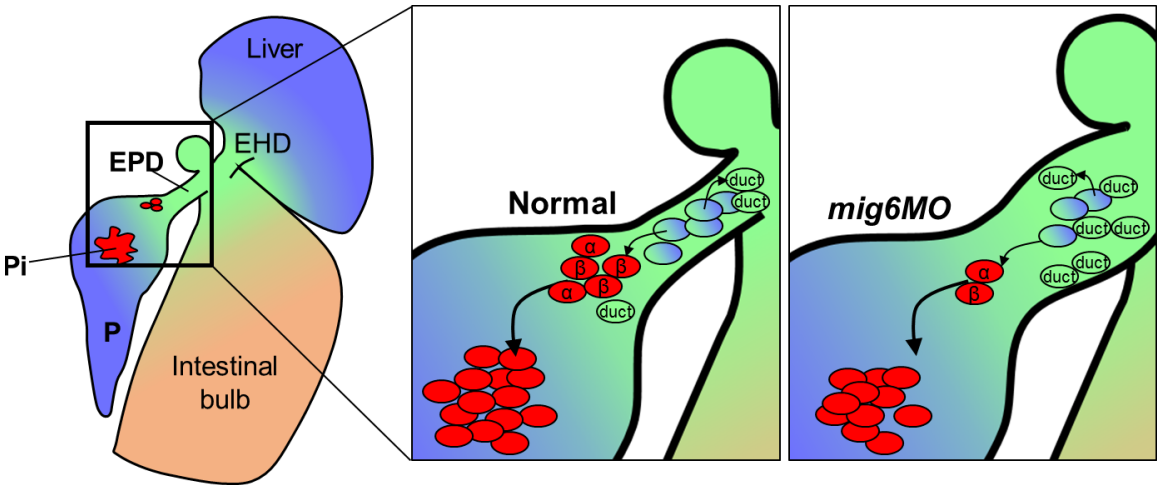


Figure 2-12. Schematic depicting proposed role for *mig6* as a switch for EPD progenitor cell differentiation.

If, by knocking down *mig6*, we have consequently blocked Notch signaling pathways, our findings and conclusions about the *mig6* agree with Notch signaling literature. Ectopic overexpression of Notch1 in the exocrine pancreas showed prevention of exocrine and endocrine differentiation of pancreatic progenitor cells, leaving them in an undifferentiated state [199-201]. Conversely, in studies using *Notch1/2* knock out mice and studies using zebrafish embryos treated with a Notch inhibitor, it was

demonstrated that blocking Notch signaling results in premature differentiation of multipotent progenitor cells into endocrine cells [202, 203].

Lastly, our results do not exclude the possibility that *mig6* does have an effect on regeneration after a β cell insult. However, because *mig6* was knocked down for most of embryogenesis, its developmental phenotype may have concealed any changed regenerative capacity that could be observed if *mig6* was conditionally knocked out after a pivotal period in pancreas formation. Such a model could uncover protective or degenerative mechanisms to progenitor cells after β cell stress. The role of *mig6* in development is crucial to pancreas development and other organogenesis, but after the onset of diabetes, it could be a promising target of endocrine cell differentiation and restoration of β cell mass.

2.6 Materials & Methods

Animal maintenance

Zebrafish were raised under standard laboratory conditions at 28°C. All zebrafish transgenic lines have been published previously [195]. Transgenic lines used are listed in **Table 2-1**. All lines were maintained and used in accordance with the "Guidelines for Use of Zebrafish in the NIH Intramural Research Program". All animal procedures were conducted following OLAW guidelines, with the approval of the IU Institutional Animal Care and Use Committee.

Genome editing

The following antisense morpholino (MO) (Gene Tools, LLC) was injected into 1-cell stage embryos: *mig6* MO (targeting NM_001083570), 5'-

ATTTCCTGGCCTGAATGCGAACCGA (0.25 ng and 0.50 ng), targeting the ATG site in exon 2. 100 pg *H2BRFP* mRNA was co-injected into zygotes along with *mig6*MO.

We followed a published protocol for targeted CRISPR mutagenesis. We used Benchling online software to design *mig6* sgRNA targeting 5'-GGCAGCCACTGGCAT-AGCA in exon 2 (#1) and 5'-AGTTTCCGAATCTTGCTCC in exon 4 (#2). *Mig6* sgRNA #1 was injected in combination with Cas9 NLS (NEB) into 1-cell stage embryos of the *Tg(ptf1a:GFP)*, and injected G_0 larvae were raised to adulthood. Primers flanking each site were designed to amplify a 290 bp (#1) and a 249 bp (#2) PCR product and are listed in **Table 2-2**. Genomic DNA extraction was performed using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). Successful CRISPR mutagenesis was indicated by a change in PCR amplicon size, visualized by agarose gel electrophoresis.

Table 2-1. Zebrafish transgenic lines

Name	**Use**	**Reference**
Tg(ins:CFP-NTR)	β cell ablation	Curado, S. et al., 2008 [172]
Tg(ptf1a:GFP)	exocrine and neural marker	Anderson, R.M. et al., 2013 [204]
Tg(nkx2.2:GFP)	duct marker	Ye, L et al., 2016 [182]
Tg(gcga:GFP)	α cell marker	Pauls, S. et al., 2007 [205]
Tg(ins:dsRed)	β cell marker	Anderson, R.M. et al., 2009 [206]

Table 2-2. Primers for zebrafish CRISPR mutation determination

Name	**Target gene**	**Sequence (5'→3')**
gRNA #1 *sense* primer	*mig6*	GGAGCATTTCCTGGCCTGAATG
gRNA #1 *antisense* primer		ACGCCAATCAGACACATTTGCT
gRNA #2 *sense* primer		CACCAAACCCCTTCCCCCAAT
gRNA #2 *antisense* primer		CTCGCCTTCTGTTGTTGTTGCT

β cell ablation and regeneration

Zebrafish embryos were collected and cultured in standard conditions at 28°C in egg water supplemented with 4 mM 1-phenyl 2-thiourea (PTU) to inhibit melanophore formation. We analyzed regenerated β cells in the control and *mig6MO*-injected embryos using targeted conditional cell ablation using *Tg(ins:CFP-NTR)*. 7.5mM metronidazole (MTZ) in egg water was added at 3 dpf and washed out at 4 dpf, allowing one day for recovery (See **Figure 2-9**).

Label retaining cell assay

100 pg *H2BRFP* mRNA was co-injected into zygotes. Dilution of the dye with every mitotic cell division allowed us to distinguish cells that were positive for the *H2BRFP* dye versus cell that were negative for the *H2BRFP* dye. Cells that differentiated early from the dorsal bud became quiescent sooner than those that undergo multiple round of mitosis from the ventral bud to make acinar and duct tissue. *H2BRFP*-positive and *H2BRFP*-negative β cells were counted as described in the following section.

Immunofluorescence and *in situ* hybridization

Whole mount immunofluorescent staining, and cell analysis were performed as previously described [207]. For immunofluorescence, the following antibodies were used: chicken α-GFP (Aves), guinea-pig α-insulin (Invitrogen), mouse α-glucagon (Sigma), mouse α-2F11 (Abcam), and rabbit α-dsRed (Takada). Alexa Fluor-conjugated antibodies (Life Technologies) were used for visualization, and DAPI or TO-PRO was used to visualize nuclei. Additional details are provided in **Table 2-3**.

For whole mount in situ hybridization (ISH), antisense probes were transcribed from DNA templates that were generated by PCR from cDNA using primer sets that

amplify *mig6, fabp10, insulin,* or *sox9b* (**Table 2-4**). RNA with the T7 or T3 promotor was labeled with the DIG RNA Labeling Kit (Roche #11175025910). Gene expression was visualized with BM Purple (Roche #11442074001) or VectorRed (Vector Labs #SK5100) for fluorescent ISH (fISH).

For Z-stack images and cell quantifications, embryos were imaged on LSM 700 confocal microscope (Zeiss) and fluorescence and brightfield images were acquired on M205 FA whole mount epifluorescence dissecting microscope (Leica). All image processing and analysis was performed using ImageJ software.

Statistical analysis

All data are presented as means ± SD unless stated otherwise. The Student's t-test (unpaired, two-tailed unless stated otherwise) or ANOVA was performed using GraphPad Prism software (La Jolla, CA, USA) to detect statistical differences. $p < 0.05$ was considered statistically significant.

Table 2-3. Antibodies used for zebrafish immunofluorescence staining

	Name	**Species**	**Dilution**	**Company**
Primary	anti-GFP	chicken	1:500 or 1000	Aves Labs #GFP-1020
	anti-insulin	guinea pig	1:100	Invitrogen #180067
	anti-glucagon	mouse	1:100	Sigma #G2654
	anti-dsRed	rabbit	1:500	Takada #632496
	anti-2F11	mouse	1:50	Abcam #ab71286
	anti-DIG AP	sheep	1:4000 (1:100 fISH)	Roche #12486522
Secondary	AlexaFluor 405	mouse, rabbit	1:250	Life Technologies #31553, #31556
	AlexaFluor 488	guinea pig, chicken	1:250	Life Technologies #A1073, #A11039
	AlexaFluor 555	rabbit, mouse, guinea pig	1:250	Life Technologies #A21428, #A31570, #A21435
	TO-PRO 647	-	1:500	Life Technologies #T3605

Table 2-4. Primers used for *in situ* hybridization

Gene name	**Abbreviated name**	**Strand**	**Sequence (5'→3')**
mitogen-inducible gene 6	*mig6*	sense	ATCTGACCGCATCAGTCCTC
		antisense + T7	gcatgcatgcattaatacgactcactatagggagaCGTCGTTTTTCGTTTGCTTT
		antisense	CGTCGTTTTTCGTTTGCTTT
		sense + T3	gcataattaaccctcactaaagggagaATCTGACCGCATCAGTCCTC
insulin a	*insa*	sense	GTCAATCGGGGGAGAAGAAG
		antisense + T7	GCtaatacgactcactataggTCACAAACATGATTGCCAGTG
SRY (sex determining region Y)-box 9b	*sox9b*	sense	ACACTCCGGTCAGTCTCAGG
		antisense + T7	taatacgactcactatagggTTGCTGTGGAGTGCAAAAAC
fatty acid binding protein 10	*fabp10*	sense	GGATCCGACGACTTTGTGTT
		antisense + T7	taatacgactcactataggGATGTTTGACCGGTACAGTGTTTATTA
T3 or T7 promoters designated by lower case characters			

CHAPTER 3. MECHANISTIC REGULATION OF MIG6 ACTIVITY

3.1 Summary

T1D is caused by autoimmune-mediated destruction of insulin-producing β cells. Following β cell injury, the pancreas attempts to launch a cellular repair and regenerative program, yet it fails to completely restore functional β cell mass. One component of the regenerative program is epidermal growth factor receptor (EGFR) signaling. Upon irreparable β cell damage, EGFR signaling is dampened, disrupting attempts to restore functional β cell mass and maintain normoglycemia. We previously demonstrated that the negative feedback inhibitor of EGFR, Mitogen-inducible gene 6 (Mig6), is induced by the pro-inflammatory cytokines central to the β cell destruction in T1D. We also established that pro-inflammatory cytokines suppress EGFR activation, and siRNA-mediated suppression of Mig6 restores EGFR signaling.

Recently, Mig6 was reported to be phosphorylated and activated by Src kinase. Interestingly, Src increases cell proliferation by activating receptor tyrosine kinases, such as EGFR, yet it is also implicated in the production of reactive oxygen species, including nitric oxide (NO). We hypothesized that pro-inflammatory cytokines activate Src kinase, and that along with NO, disrupt EGFR repair mechanisms through the induction and activation of Mig6. To test this hypothesis, we treated 832/13 cells independently with cytokines and high glucose and identified that these conditions increase the activation of Src. We confirmed these results *ex vivo* in mouse islets. In the 832/13 cells, Src inactivation may protect EGFR signaling from the detrimental effects of cytokine exposure, but the role of nitric oxide (NO) was inconclusive. Our work suggests that Src

kinase may be involved in the progression to T1D by activating Mig6, and may be a component of Mig6 regulation that can be targeted to preserve β cell mass in T1D.

3.2 Introduction

In the progression to T1D, immune cells release pro-inflammatory cytokines into the injured β cell. Pro-inflammatory cytokines are well established as mediators of β cell damage [208, 209], a concept recapitulated *in vitro* [210]. Previous work has determined that among all the cytokines released during the immune response, only TNF-α, IL-1β, and IFN-γ are required to produce the full inflammatory response in human islets [80]. The cytokines TNF-α and IL-1β stimulate their respective receptors and signal downstream to release NFκB from its inactivated complex with IκB; NFκB then translocates to the nucleus [211] to activate inducible NO synthase (iNOS) transcription, and subsequent NO production [212, 213]. NO is essential for β cell dysfunction and apoptosis [214]. Beyond activating NFκB signaling and producing NO, cytokines dampen EGFR activation in 832/13 cells [105], but the precise mechanism remains unknown.

The mechanism(s) through which cytokines induce Mig6 expression, like EGFR inactivation, are also unclear. However, a recent report documented that activated Src "primes" Mig6 for EGFR inhibition (**Figure 3-1**) [113]. Src, a non-receptor tyrosine kinase of the Src family kinases, has been implicated in proliferation, survival, apoptosis and differentiation [118]. Src is activated by receptor tyrosine kinases, like EGFR, as well as other stimuli that are altered in diabetes pathogenesis, such as G-protein coupled

receptors, TGF-β, and ROS [119-121]. In addition, the cytokines IL-1β and TNF-α individually induce Src activation [123, 124].

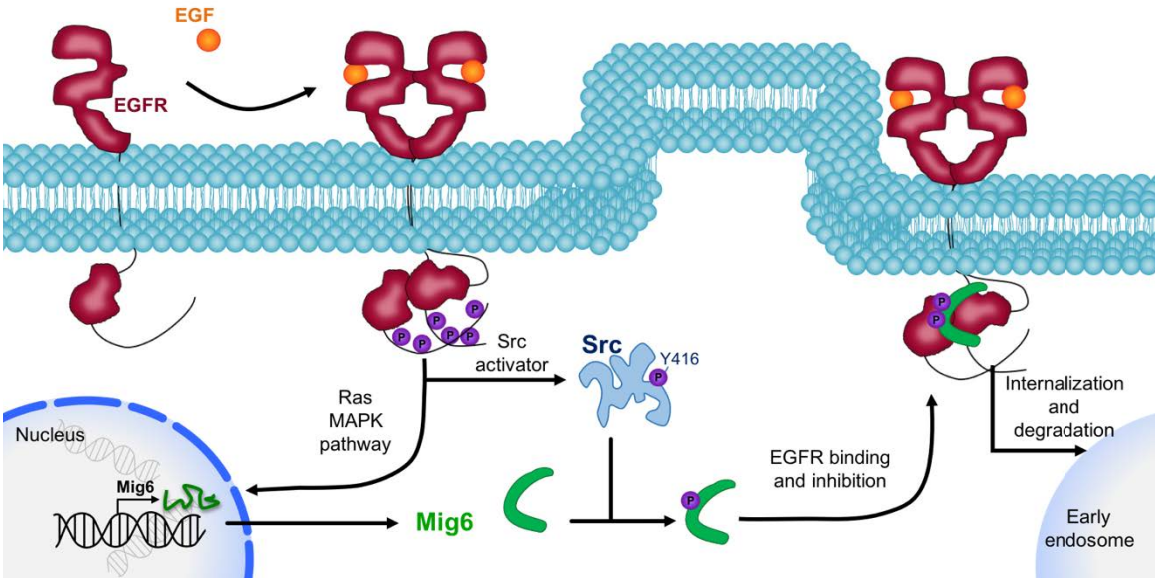


Figure 3-1. Proposed regulation and phosphorylation of Mig6 by a Src kinase.

3.3 Results

Src kinase is phosphorylated under both high glucose and cytokine treatments

It was previously demonstrated that treatment with IL-1β and TNF-α increased Src activation in human brain (T98G) and muscle (HTSM) cells, respectively [123, 124]. To assess the effect of cytokines and diabetogenic conditions in the 832/13 cells, we treated with RPMI media containing either 16.7 mM glucose (high glucose) or a pro-inflammatory cytokine 'cocktail' of IFN-γ, IL-1β and TNF-α (hereafter referred to as cytokines). High glucose increased Src kinase activation, as measured by phosphorylation of tyrosine residue 416 (**Figure 3-2 A**). Cytokine treatment also increased Src phosphorylation in 832/13 cells (**Figure 3-2 B**). Cytokines increase Mig6 expression in

human and rodent islets [105]. To find similarities in physiological function, we treated isolated mouse islets with cytokines. In islets, 16 h cytokine treatment increased Src kinase phosphorylation (**Figure 3-2 C**). We hypothesized that Src activation increased Mig6 expression, but the mechanism is still unclear.

Next, to assess the net and cumulative phosphorylation of Src, we inhibited phosphatase activity to promote Src activation under cytokine conditions. Cytokine pretreatment increased the cumulative phosphorylation of Src in both 832/13 cells (**Figure 3-3 A, C**) and isolated mouse islets (**Figure 3-3 B, D**).

Src activation was not increased independently of EGFR, but Src inhibition may restore cytokine-impaired EGFR signaling

EGFR has been shown to activate Src kinase in various cancer cell lines [215]. Thus, 832/13 cells treated with AG1478, a selective, irreversible EGFR inhibitor, was expected to decrease Src kinase activation. Instead, we observed unchanged Src activation (**Figure 3-4 A, B**). Surprisingly, when we treated cells with the Src inhibitor, PP1, EGFR signaling increased in cytokine-treated groups (**Figure 3-4 C**), suggesting a role for Src in cellular stress mechanisms, although this idea was not directly tested.

Src inhibition activates *Nos2*, but NO decreases the net cumulative phosphorylation and activation of Src kinase

Aside from its supposed Mig6 'priming' role, we speculated that Src may also be important for gene expression, thus 832/13 cells were treated with several common Src kinase inhibitors. However, treatment with SU6656, PP1, and PP2 had no effect on *Mig6* expression (**Figure 3-5 A**). Src is activated by receptor tyrosine kinases, like EGFR, as well as other stimuli such as ROS [119-121]. We hypothesized that cytokines induce Src

activation by activating the NFκB signaling cascade to produce ROS such as nitric oxide (NO). Hence, we treated 832/13 cells with Src inhibitors to assess the role of Src in NO production. We report that with Src inhibition, *Nos2* (mRNA precursor to iNOS) expression is increased (**Figure 3-5 B**) and pretreatment with PP1 allowed for increased cytokine-mediated *Nos2* expression (**Figure 3-5 C**). In a reciprocal study, we evaluated the implications of increased NO production in the 832/13 cells. We treated cells with the NO donor DPTA/NO and quantified the change in Src activation. Interestingly, NO decreased the phosphorylation of Src (**Figure 3-5 D**), suggesting that in 832/13 cells, NO and Src may complement each other in regulatory mechanism.

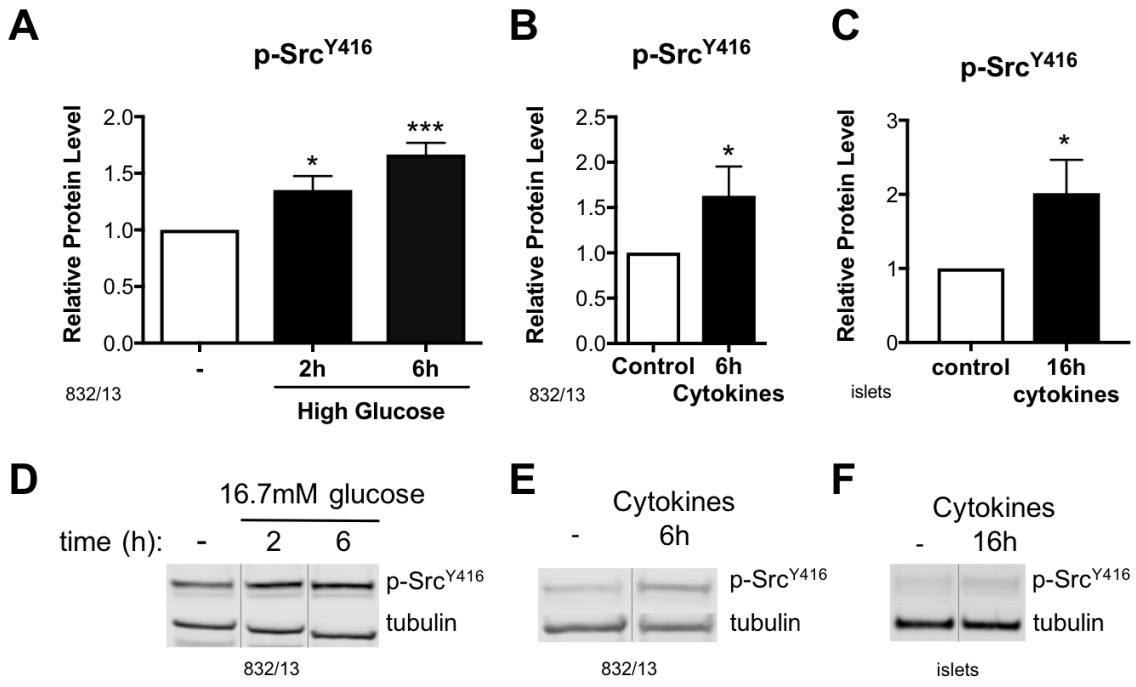


Figure 3-2. High glucose and pro-inflammatory cytokines induce Src kinase phosphorylation in 832/13 cells and isolated mouse islets. Rat insulinoma 832/13 cells were starved 24h in 2.5mM glucose and treated with 16.7mM glucose media (high glucose) or 50U/ml IL-1β, 1000U/ml TNFα, and 1000U/ml IFN-γ (cytokines) for specified times and cell lysates were collected for western blot analysis. Src activation, as measured by phosphorylation of the active site at tyrosine-416, increases upon (**A**) exposure to 16.7mM glucose for 6h or (**B**) exposure to cytokines for 6h; *n = 4-5*. (**C**) Isolated mouse islets were treated with cytokines for 16h; *n=4*. (**D-F**) Representative images of western blots of graphs A-C. Data are reported as protein levels relative to noncytokine-treated, non-pV-treated group and normalized to tubulin. **, p<0.05 vs. non-treated; ***, p<0.001 vs. non-treated. One-way ANOVA (A) or Student's t-test (B,C).*

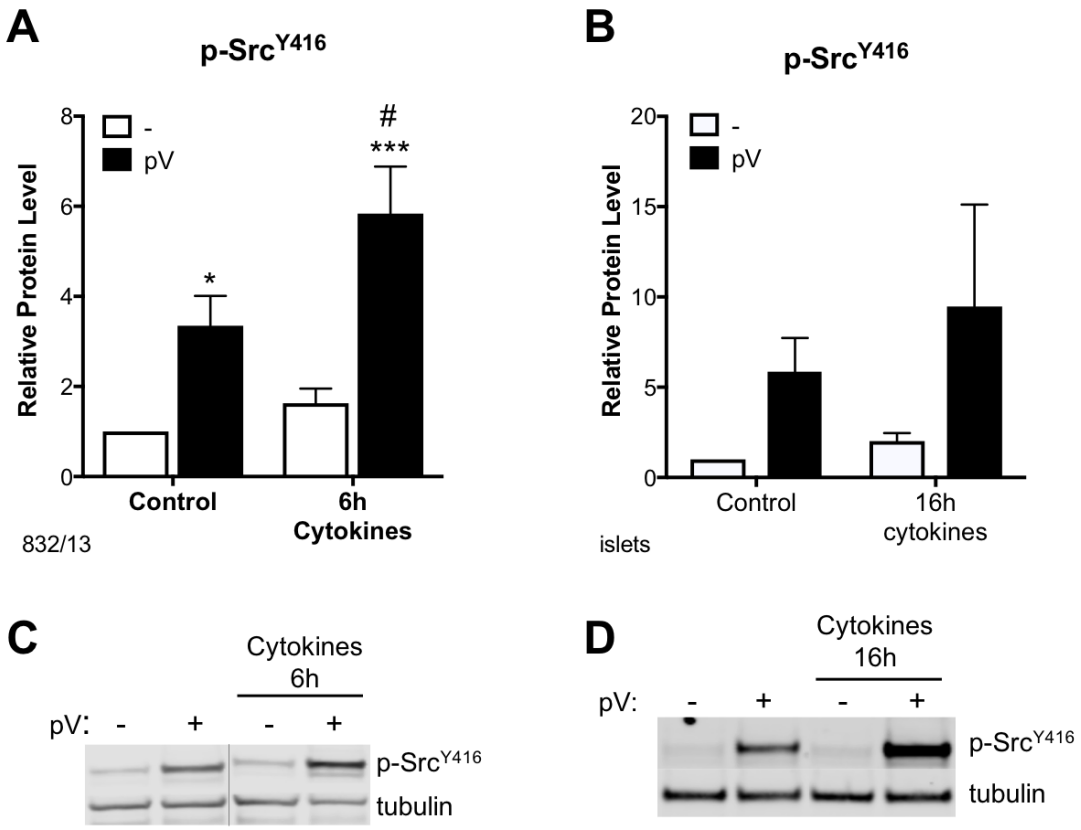


Figure 3-3. Inhibition of phosphatase activity permits heightened cytokine-mediated Src activation. Rat insulinoma 832/13 cells or isolated mouse islets were starved for 24 h in 2.5mM glucose and treated with cytokines. Cell lysates were collected for western blot analysis (**A**) After 832/13 cells were treated with cytokines for 6h, pervanadate (pV) was used to inhibit phosphatase activity; *n=4-5. *, p<0.05 vs non-treated; ***, p<0.001 vs non-treated;* $^{\#}$*, p < 0.05, control vs cytokine pV-treated in 2-way ANOVA.* (**B**) Isolated mouse islets were treated with 16 h cytokines and pV was used to inhibit phosphatase activity; *n=2-3.* (**C, D**) Representative images of western blots of graphs A and B.

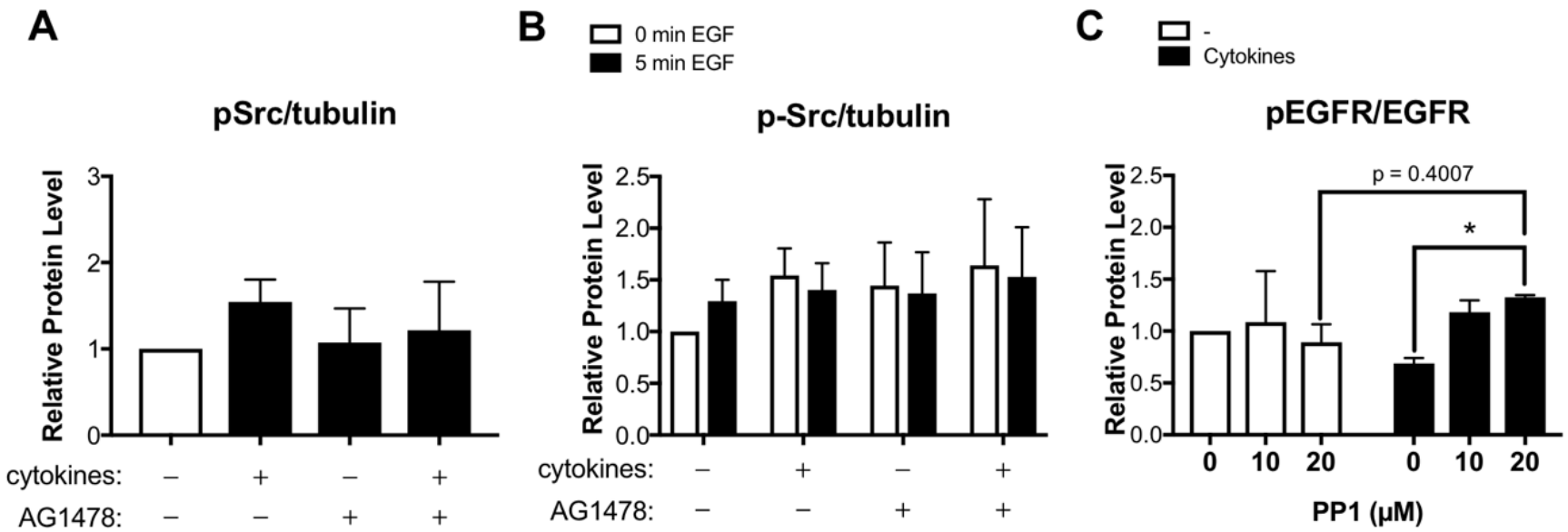


Figure 3-4. EGFR inhibition did not affect cytokine-mediated Src activation, but Src inhibition may restore cytokine-impaired EGFR signaling. (**A**) 832/13 cells were treated with cytokines and/or the EGFR inhibitor, 250μM AG1478 for 16 h, and (**B**) starved for 2h in 2.5mM glucose and stimulated with 10 ng/ml rrEGF for 5 min. *n = 3*. (**C**) 832/13 cells were pretreated with Src inhibitor PP1 for 30 minutes at 10 and 20μM, then exposed to cytokines for 6h. *n = 4; *, p<0.05 0 vs. 20μM PP1 cytokine-treated group, 2-way ANOVA.*

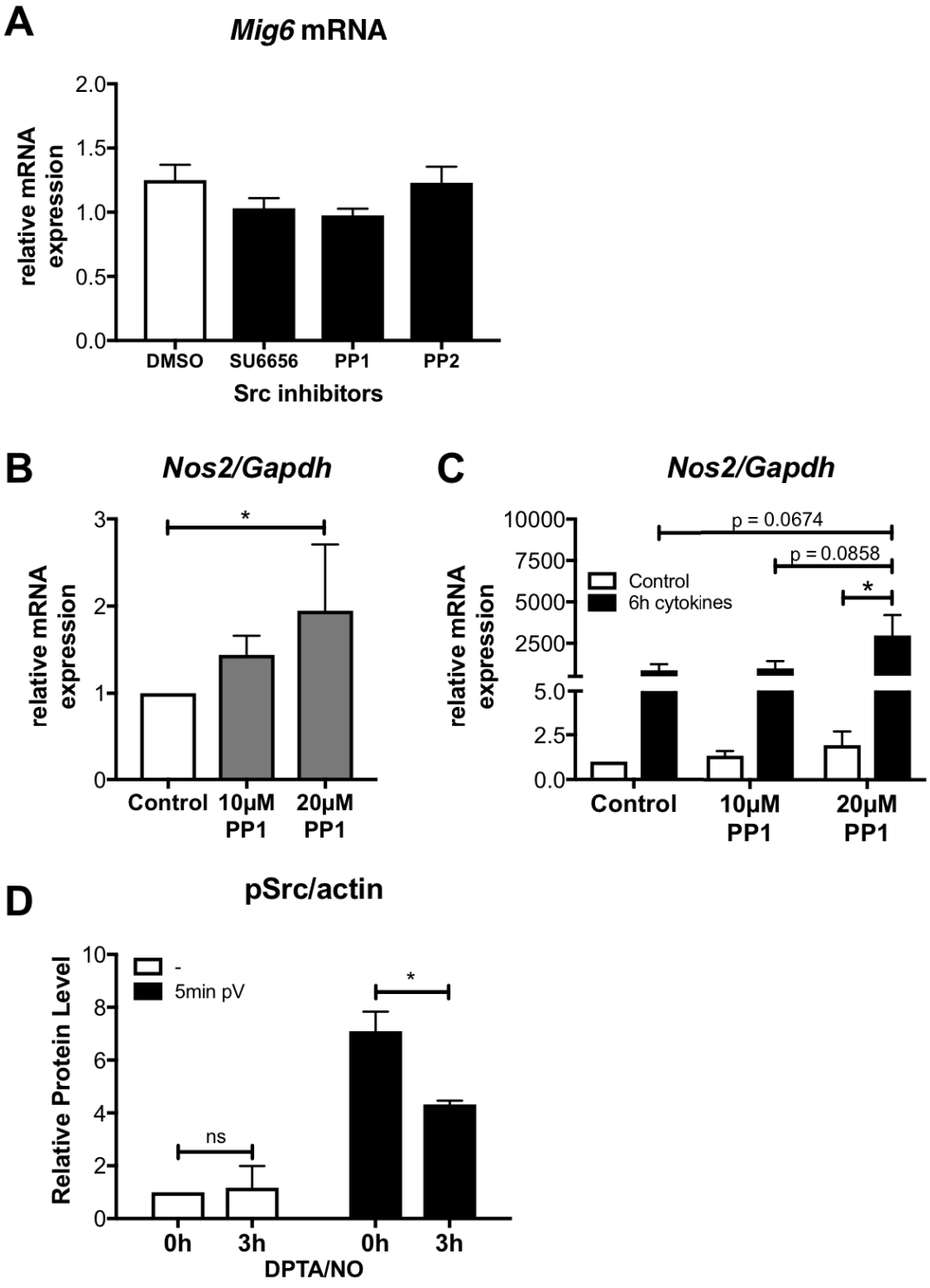

A
Mig6 mRNA
relative mRNA expression
2.0
1.5
1.0
0.5
0.0
DMSO
SU6656
PP1
PP2
Src inhibitors
B
Nos2/Gapdh
relative mRNA expression
3
2
1
0
*
Control
10μM PP1
20μM PP1
C
Nos2/Gapdh
relative mRNA expression
10000
7500
5000
2500
5.0
2.5
0.0
p = 0.0674
p = 0.0858
*
Control
6h cytokines
Control
10μM PP1
20μM PP1
D
pSrc/actin
Relative Protein Level
10
8
6
4
2
0
-
5min pV
ns
*
0h
3h
0h
3h
DPTA/NO

Figure 3-5. Src inhibition increases *Nos2* expression and NO donor decreases cumulative phosphorylation and activation of Src kinase. (**A**) 832/13 cells were treated with SU6656 (10μM), PP1 (20μM), and PP2 (20μM) for 30 min. Relative expression normalized to *Gapdh* was determined by qRT-PCR; *n=7*. (**B**) 832/13 cells were treated with 10 or 20μM PP1 for 30 minutes *(n=3-5; *, p<0.05, One-way ANOVA)* or (**C**) pre-treated with PP1 for 30 minutes, then exposed to 6h cytokines. *Mig6, Nos2* and *Gapdh* mRNA levels were determined by qRT-PCR. *n=4-5.* (**D**) 832/13 cells were treated with 3h DPTA/NO then phosphatase activity was inhibited with 5min 50μM pV. *n=2. *, p<0.05, 2-way ANOVA.*

3.4 Preliminary Results

Phosphorylation of Mig6 in cytokine-treated rat insulinoma cells

Recently, Mig6 was discovered to be phosphorylated at tyrosine residues 394 and 395 [113] and this portion of the Mig6 protein interacts with the EGFR kinase domain to inactivate downstream signaling [114]. To study the phosphorylation of Mig6, we commissioned a phospho-Mig6^{Y394}, phospho-Mig6^{Y395} and phospho-Mig6^{Y394Y395} antibody from 21st Century Biochemicals. Unfortunately, with this antibody, we could not detect tyrosine phosphorylation of Mig6 under cytokine, pV, or EGF- treatment (*data not shown*). Furthermore, mass spectrometry analysis indicated that Mig6 was phosphorylated on serine/threonine residues under cytokine treatment in 832/13 cells (*data not shown, unpublished*).

β cell mass in Src whole-body knock out mice

We determined β cell mass in Src whole-body knock out (Src KO) mouse pancreas sections by immunohistochemical staining of insulin. Preliminarily, there was no difference in β cell mass between wild type and Src knock out mice, but low statistical power may mask any trend of increased β cell mass (**Figure 3-6**). The Src KO mice exhibit severe bone fragility and would not be a good model of β cell stress. For this reason, we did not challenge the Src whole-body knock out mice to streptozotocin (STZ)-induced β cell injury. We do speculate that a pancreas-specific knock out of Src would allow us to study the effect of Src on β cell mass after β cell injury.

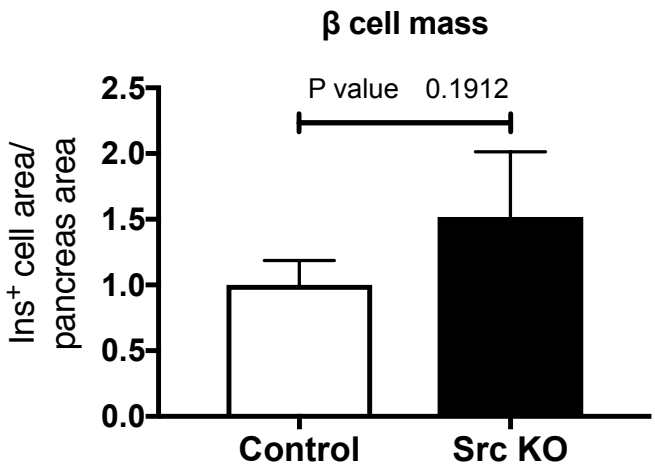


Figure 3-6. Preliminarily, β cell mass in not changed in Src KO mice. Quantified islet area relative to total pancreatic area was calculated from immunohistochemical staining for insulin in pancreatic sections of control and Src KO mice, *n = 3*, each group. *Student's t-test, one-tailed.*

3.5 Discussion

Regulation of EGFR signaling via ancillary mechanisms like Mig6 circumvents concerns of targeting EGFR directly and the possibilities of tumor formation. To properly assess the potential of Mig6 as a therapeutic target, we must understand its endogenous regulatory mechanism, which is currently poorly defined. Src kinase was suggested to mediate phosphorylation of Mig6 at residues Y394 and Y395, but in this study, we were unable to confirm or refute this observation. We report that diabetogenic conditions (high glucose and cytokines) induce activation of Src kinase and increase the maximum phosphorylation threshold of Src kinase in both 832/13 cells and isolated mouse islets, implying that during the progression of diabetes and undiagnosed/unmanaged hyperglycemia, Src is activated. From our data, it appears Src is detrimental to EGFR signaling in 832/13 cells as Src inhibition resulted in increased p-EGFR in cytokine-exposed conditions. Elsewhere, in high glucose conditions, Src inhibition protected against stress-induced EGFR transactivation and prevented nephrotic complications of diabetes [216]. Our current study did not assess transcriptional changes in EGFR, but we cannot dismiss the notion that the increased basal EGFR could be due to increased transcription of EGFR and higher numbers of receptors expressed on the cell surface, rather than increased EGFR activation *per se*. To assess the role of Src in cytokine-mediated EGFR inhibition, we would need to evaluate Src inhibition on EGFR ligand-dependent activation.

Analysis of Mig6 phosphorylation was inconclusive, and our studies may have been limited by the assumption that it was phosphorylated on tyrosine residues in the 832/13 cell line, as in other cell types. For instance, checkpoint kinase 1 (Chk1) was

shown to phosphorylate Mig6 at serine 251 in human cell lines [111]. Although it is unknown how phosphorylation of serine 251 control aspects of Mig6 function, we wonder if Mig6 may function in concert with serine/threonine kinases (STKs), which have been associated with anti-proliferative/pro-apoptotic mechanisms and cell cycle regulation [217, 218]. We did assess transcriptional regulation of Mig6 by Src kinase by inhibiting Src, but there was no significant change in Mig6 expression. Src inhibition did increase iNOS expression, and we observed that Src inhibition tended to allow for increased cytokine-induced NO production. However, treatment with a NO donor decreased the activation of Src kinase (Y416). Src may serve as a buffer for cytokine-mediated NO production and cytokine induce it as a self-check mechanism.

Most of the studies reported here were conducted with Src inhibitors, but perhaps the expression of constitutively active Src kinase would directly inform a role in Mig6 expression or protein. In addition, what this study did not elucidate was the effect of EGFR inhibition on modulation of Src kinase on other phosphorylation sites, such as on Y527, which would aide in Src kinase activation. The activation of Src is facilitated by phosphorylation of Y416 as well as dephosphorylation on Y527 [219].

From these data, we can conclude that Src kinase in activated under diabetogenic conditions, such as high glucose and cytokines, and its activation facilitates cytokine-mediated NO production. Further studies would reveal the impact of increased activated Src kinase on cytokine-mediated EGFR activation or possible transactivation. In addition, an antibody that could detect Mig6 phosphorylation on Y395/Y394 would delineate whether or not Src activation increases p-Mig6. Nonetheless, our study contributes data

of mitogenic signaling control mechanisms in the 832/13 cells, that may be useful as the regulatory mechanism of Mig6 becomes more apparent.

3.6 Materials & Methods

Animal maintenance

All animals were maintained and used in accordance with protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee, following the *Guide for the care and use of laboratory animals*, Eighth edition (2011). Mice were maintained in a standard 12-hour light-dark cycle and provided unrestricted access to water and a standard rodent chow. Wild type C57Bl/6J mice were used for islet experiments. Src knock out mouse tissue was a gift from Dr. Fred Pavalko (Indiana University). Extract-N-Amp Tissue PCR kit (Sigma-Aldrich, St Louis, MO, USA) was used for DNA extraction.

Histological studies

Immunostaining of pancreatic sections was performed as previously described [105]. β cell mass was determined by staining for insulin (Invitrogen #180067) and calculating insulin-positive area relative to total pancreas area.

Islet experiments

Mouse islets were isolated and cultured as previously described [106, 220] for protein and mRNA analysis. Groups of 50-100 islets from wild type mice were pretreated with a pro-inflammatory mouse cytokine cocktail (1000 U/ml TNF-α, 50 U/ml IL-1β, and 1000 U/ml IFN-γ; Prospec, East Brunswick, NJ) and treated with 50 μM pervanadate (pV) for 5 min in 11.1 mM glucose RPMI.

Cell experiments

INS-1-derived 832/13 rat insulinoma cells were cultured as previously described [221]. A starvation medium (RPMI 1640 containing 2.5 mmol/l glucose and 0.1% BSA) was used for EGF stimulation experiments. For cytokine plus pV experiments, 832/13 cells were pretreated with a pro-inflammatory rat cytokine 'cocktail' (1000 U/ml TNF-α, 50U/ml IL-1β, and 1000 U/ml IFN-γ; Prospec, East Brunswick, NJ) for 16 hours, starved for 2 hours and treated with 50 μM pV (R&D Systems, Minneapolis, MN, USA) for 5 min. For nitic oxide experiments, 832/13 cells were treated with 200 μM DPTA/NO (Cayman Chemical, Ann Arbor, MI, USA) for 3 hours. For EGFR inhibition experiments, AG1478 was added at 20 μM. The Src inhibitors used were SU6656 (10 μM), PP1 (10 and 20 μM), and PP2 (20 μM) for 30 min.

Immunoblot analysis

Immunoblot analysis was performed as previously described [106]. Phosphorylated protein levels were normalized to total protein levels, and total (i.e. non-phosphorylated) protein levels were normalized to tubulin or GAPDH protein levels. Antibodies are listed in **Table 3-1**.

Quantitative RT-PCR analysis

RNA from 832/13 cells, and mouse, rat, and human islets was isolated using RNeasy Mini or Micro kits (Qiagen, Valencia, CA, USA). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan assays (Applied Biosystems) were used to obtain C_T values and the $\Delta\Delta C_T$ method was used to calculate the relative quantities of *mig6*, *gapdh*, or *NOS2* mRNA. Primer sequences were described previously [222]. PCR reactions were

performed in triplicate for each sample from at least three independent experiments and normalized to *Gapdh* mRNA levels.

Table 3-1. Antibodies used for immunoblotting

Name	**Vendor, catalog number**	**Dilution**	**Solvent**
anti-phospho-EGFR (Y1068)	Cell Signaling, #3777	1:1000	Nacalai USA Signal Enhancer Hakari 250
anti-EGFR	Sigma-Aldrich, #E3138	1:1000	Nacalai USA Signal Enhancer Hakari 250
anti-γ-tubulin	Sigma-Aldrich, #T6557	1:2500	PVP
anti-Mig6	Santa Cruz, #D-1 21st Century Biosciences,	1:250 1:200	PVP PVP
Anti-Src kinase	Cell Signaling	1:1000	PVP
Anti-phospho-Src(Y416)	Cell Signaling	1:1000	PVP
Anti-actin	MP Biomedicals #691002	1:2500	PVP

Statistical analysis

All data are presented as means ± SEM. Protein and mRNA data were normalized to control conditions and presented as relative expression. The Student's t-test (unpaired, two-tailed unless stated otherwise) or ANOVA (with Bonferroni post hoc tests) were performed using GraphPad Prism software (La Jolla, CA, USA) to detect statistical differences. $p < 0.05$ was considered statistically significant.

CHAPTER 4. MIG6 ACCELERATES THE PROGRESSION TO DIABETES BY BLOCKING ENDOGENOUS EGFR REGENERATIVE MECHANISMS

4.1 Summary

Type 1 Diabetes (T1D) is caused by autoimmune-mediated β cell destruction. Following β cell injury, the pancreas attempts to launch a cellular repair and regenerate program, yet it fails to completely restore functional β cell mass. One component of the regenerative program is epidermal growth factor receptor (EGFR) signaling. However, upon irreparable β cell damage, EGFR signaling is dampened, disrupting attempts to restore functional β cell mass and maintain normoglycemia. We have previously demonstrated that the negative feedback inhibitor of EGFR, Mitogen-inducible gene 6 (Mig6), is induced by the pro-inflammatory cytokines central to the autoimmune-mediated β cell destruction. We also established that pro-inflammatory cytokines suppress EGFR activation, and siRNA-mediated suppression of Mig6 restores EGFR signaling. Thus, we hypothesized that pro-inflammatory cytokines induce nitric oxide production and that in turn induced Mig6, disrupting EGFR repair mechanisms. We determined that NO induces Mig6, attenuating EGFR signaling, and NO synthase inhibition blocks the cytokine-mediated induction of Mig6, thereby restoring cytokine-impaired EGFR signaling. To that end, we treated mice lacking pancreatic Mig6 and wild-type mice with a streptozotocin (STZ) to induce β cell death and diabetes in a way that mimics the onset and progression of T1D. Whereas STZ-treated wild-type mice became hyperglycemic and had reduced β cell mass, STZ-treated Mig6 PKO mice remained euglycemic and glucose tolerant due to preserved β cell mass. β cell

proliferation seems to be involved in this preservation. Our work suggests that Mig6 is a promising target to preserve β cell mass before overt T1D.

4.2 Introduction

Type 1 Diabetes (T1D) is a progressive disease characterized by autoimmune-mediated destruction of the pancreatic insulin-secreting β cells. Multiple human and animal studies support the concept that progression to overt diabetes is accompanied by both β cell dysfunction and destruction [100, 105]. β cell destruction in T1D can occur through a series of immunological attacks and recoveries, termed "relapsing-remitting" [33]: a process which gradually reduces functional β cell mass until symptoms of hyperglycemia are noticed and diabetes is diagnosed. During the progression to diabetes, immune cells such as macrophages are recruited and promote inflammation by releasing pro-inflammatory cytokines into the islet [10], which activates stress activated kinases, as well as NFκB signaling and iNOS expression [214, 223]. Such signaling events lead to increased endoplasmic reticulum (ER) stress, increased apoptosis, and decreased β cell function and regeneration, all of which culminates in the loss of functional β cell mass and diabetes.

The relapsing-remitting features of diabetes extends past clinical diagnosis to the 'honeymoon phase', where residual bet cell mass provides endogenous insulin to support exogenously delivered insulin by the patient. The honeymoon phase provides an opportune time for therapeutic intervention; in fact, clinical trials for delivering immunomodulatory agents in this window are underway [224]. These therapeutic strategies, that target the immune response but neglect intrinsic cellular pathologies

within the islet, have resulted in immunological approaches that are largely ineffective in the long term. In contrast, cellular therapies can promote β cell proliferation, survival and recovery, essentially restoring β cell mass [41]. Nevertheless, translating pre-clinical successes to patients with T1D has been challenging. For example, administration of epidermal growth receptor (EGF) and gastrin in combination increases β cell mass and reverses hyperglycemia in NOD mice [99], but this approach has yet to translate into a viable treatment for humans.

The EGF receptor (EGFR) cascade has proven to be crucial in the regulation of pancreatic β cell mass as it regulates cell growth, proliferation, survival, and differentiation [225]. Mice lacking EGFR acquire diabetes within two weeks of birth and have impaired islet development [95], and mice expressing constitutively active EGFR have increased β cell mass [97]. EGFR signaling activates downstream effectors such as Akt, which promotes cell survival, and ERK, which increases cell proliferation [106]. Additionally, in some β cell regeneration studies, expression of EGF ligands increases [93], suggesting that this pathway is involved in an intrinsic β cell regeneration and repair program.

However, endogenous feedback mechanisms restrain EGFR activation, thereby preventing the full regenerative actions of EGFR signaling. One such mechanism is Mig6, a cellular response protein and feedback inhibitor of EGFR that has been characterized as a molecular “brake” for β cell proliferation [107, 226] and survival [105, 106]. Activation of EGFR signaling induces Mig6 in a classic feedback mechanism, suppressing kinase activity and initiating receptor endocytosis and degradation [227]. Mig6 haploinsufficient mice are protected against chemically-induced diabetes [105],

suggesting that Mig6 antagonizes β cell mass. In addition, increasing Mig6 expression with a recombinant adenoviral compromises β cell integrity and islet function [105]. Thus, Mig6 expression levels can modulate functional β cell mass.

Not only is Mig6 induced during EGFR activation, but it is also activated by other factors, such as the pro-inflammatory cytokines from the T1D milieu [105]. Pro-inflammatory cytokines are well established mediators of β cell damage [208, 228], and this concept has been verified *in vitro* [210]. Previous work has determined that of all the cytokines released during the immune response, only TNF-α, IL-1β, and IFN-γ are required to produce the full inflammatory response [80]. TNF-α and IL-1β stimulate their respective receptors and initiate a downstream signaling cascade that results in transcription of inducible NO synthase (iNOS) and consequent NO production [211-213]. NO is essential for β cell dysfunction and apoptosis [214]. Besides activation of the NFκB pathway and NO production, we have demonstrated that cytokines reduce the EGFR signaling, which can be rescued by Mig6 suppression. We aim to address the role of NO in cytokine-mediated EGFR suppression as well as *in vivo* β cell regenerative effects of Mig6 pancreas-specific knock out.

4.3 Results

NO is detrimental to EGFR signaling

Previous studies in our lab have reported that cytokines dampen EGFR phosphorylation and that Mig6 suppression by siRNA rescues this inhibition in 832/13 cells [105]. Given our findings on the relationship between NO and Mig6, we investigated the direct role of NO on EGFR signaling. As an extension of our previous

work [105], we discovered that NO alone dampens EGFR phosphorylation (**Figure 4-1 A-B**). Yet, signaling of extracellular signal-regulated kinase 1 and 2 (ERK1/2), a downstream messenger of EGFR, remains intact following treatment with NO donor, suggesting that this downstream signaling arm persists (**Figure 4-1 C**).

To further assess the role of NO in EGFR signaling, we treated 832/13 cells with the iNOS inhibitor, L-NMMA (**Figure 4-2 A**) and observed that cytokines dampen EGFR signaling as expected, but that a combinatory treatment of cytokines and L-NMMA caused an overwhelming restoration of EGFR signaling (**Figure 4-2 B**). We confirmed NO production with cytokine and NO donor/inhibitor treatments by measuring nitrite production (**Figure 4-1 D, 4-2 D**).

Cytokine-induced Mig6 expression requires NO

It is widely accepted that cytokines, the mediators of immune-cell damage, are involved in the progression to T1D [229]. Pro-inflammatory cytokines induce Mig6 expression in both human islets and 832/13 cells [105]. Here, we verify that cytokines increase Mig6 expression in the 832/13 cell line (**Figure 4-3 A**), and demonstrated that cytokine-induced Mig6 expression requires cytokine-mediated NO (Figure 4-2). Interestingly, NO alone was not sufficient to increase Mig6 expression in either a dose- or time-dependent manner (**Figure 4-3 B-C**), suggesting NO functions in a permissive manner to induce Mig6 with activated cytokine signaling. This would also imply that dampening of EGFR signaling by NO donor is independent of Mig6 feedback inhibition.

Taken together, these data indicate that NO is detrimental to EGFR signaling. By contrast, at the transcriptional level, NO itself does not induce Mig6. We maintained our impression that Mig6 activates repair mechanisms through the EGFR pathway. So, we

used an *in vivo* transgenic mouse to assess how the loss of *Mig6* affects repair mechanisms downstream of EGFR signaling.

Mig6 PKO mice have normal β cell mass and islet structure

As previously reported, whole-body Mig6 heterozygous knock out mice were protected from streptozotocin (STZ)-induced glucose intolerance, with no change in insulin sensitivity [105]. Yet, this mouse model was not pancreas-specific, and the corrections in glucose tolerance waned at 20-days post STZ injection. To directly assess Mig6's actions in the pancreas, we developed a pancreas-specific Mig6 knock out mouse (Mig6 PKO) by breeding a *Pdx*-Cre mouse model with $Mig6^{flox/flox}$ mice, developed as previously described [230]. This knock out resulted in a 50% knock down of Mig6 protein and mRNA in the pancreatic islet (**Figure 4-4 A-C**). Control and Mig6 PKO mice have no difference in β cell mass (**Figure 4-4 D**), quantified from immunohistochemical (IHC) staining of insulin as insulin-positive area relative to pancreas area. We also observed no difference in islet morphology (**Figure 4-4 E**).

Mig6 PKO mice have lower fasting blood glucose and increased glucose tolerance after STZ treatment compared to control mice

Multiple low doses (MLD) of STZ causes hyperglycemia by damaging DNA in the β cell and by inducing inflammatory mediators that infiltrate the islet, promoting β cell dysfunction and death [231, 232]. MLD-STZ treatment is an experimental model that mimics features of the autoimmune-mediated β cell destruction observed during the progression to human T1D. We subjected Mig6 PKO and control mice to MLD-STZ, then challenged them by performing glucose tolerance tests at 3-days and 20-days post STZ injection. 3-days post STZ treatment, whereas control littermates developed fasting

hyperglycemia, Mig6 PKO mice maintained fasting blood glucose comparable to pretreatment blood glucose concentrations (**Figure 4-5 A-B**). Mig6 PKO mice maintained this glycemic control to at least 20-days post STZ treatment. In addition, when given a glucose challenge, Mig6 PKO mice treated with STZ remained glucose tolerant compared to their littermate controls at 3-days post STZ treatment and this perpetuated 20-days post STZ (**Figure 4-5 C-F)**.

Mig6 PKO mice have preserved β cell mass after STZ treatment

Prolonged fasting normoglycemia and glucose tolerance suggests Mig6 PKO mice are protected against STZ injury. We again performed IHC staining for insulin on 20-days post STZ sections and normalized to pancreas area. B cell mass was significantly higher in STZ-treated MIg6 PKO mice compared to STZ-treated control mice and was not different to saline treated mice (**Figure 4-6 A-B**). At 3- and 20-days post STZ treatment, we counted the total number of established islets, omitting groups of less than 10 β cells. Because there was no change in islet architecture, we suspected that β cell proliferation played a role in the observed protection.

Ki67 and phospho-histone H3 (pHH3) are markers of cells in the cell cycle. We counted the number of Ki67-positive or pHH3-positve β cells in pancreas sections of control or Mig6 PKO mice. Following STZ injury, Mig6 PKO mice have a higher number of proliferating β cells in the early stages of recovery, demonstrated an increased proportion of Ki67-positive cells to total islet area (**Figure 4-7 A**), as well as a higher absolute number of Ki67-positive cells (**Figure 4-7 C**). Although the increased number of Ki67-positive cells persisted at 20-days post-STZ, the relative proliferative effect ceased after 20 days of recovery (**Figure 4-7 E**). There were no changes in pHH3-

positive β cells at 3-days (**Figure 4-7 B, D**) or 20-days post-STZ treatment (**Figure 4-7 F, H**). This depression in proliferative response late after β cell insult may contribute to the waning protection against hyperglycemia at 20 days recovery (**Figure 4-5**).

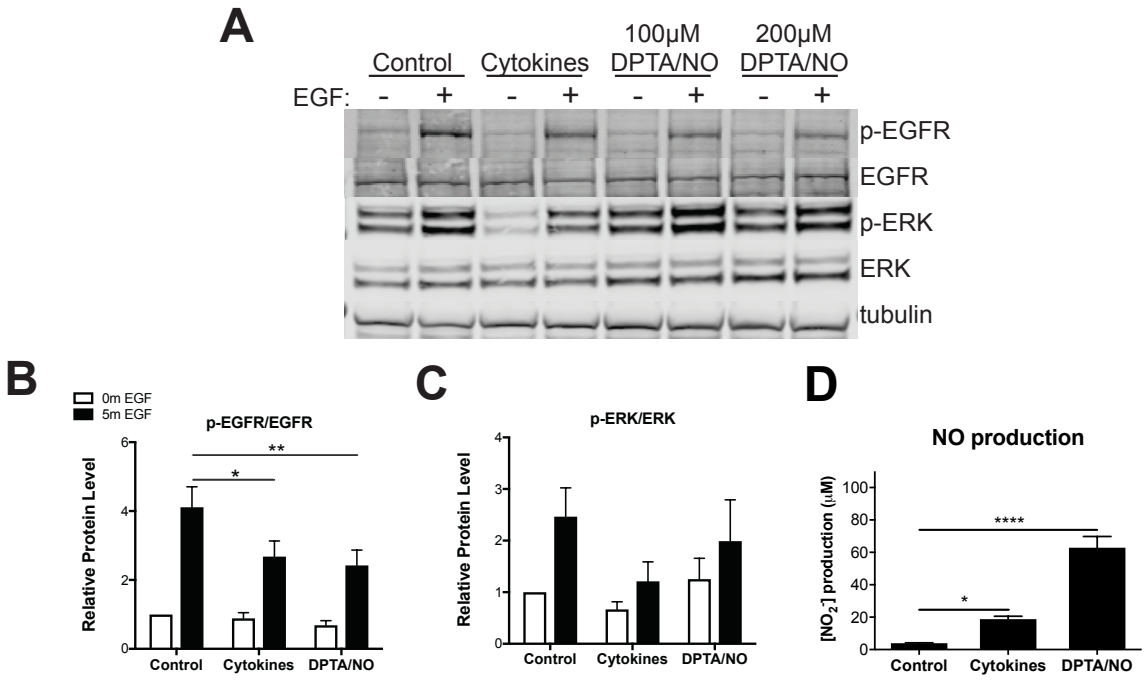


Figure 4-1. Cytokines and nitric oxide attenuate EGFR signaling. **(A)** Representative image of western blot in which 832/13 cells were treated with cytokines (TNFα, IL-1β, IFN-γ) (8h) or nitric oxide donor, DPTA/NO (4h), starved for 2h in 2.5mM glucose and stimulated with 10 ng/ml rrEGF for 5 min. Cell lysates were collected for western blot analysis to determine p-EGFR, EGFR, p-ERK, ERK, and tubulin levels. **(B, C)** Quantified p-EGFR/EGFR and p-ERK/ERK reported as protein levels relative to non-cytokine, non-EGF treated groups and normalized to tubulin. *n = 4-5; *, p<0.05 vs control EGF-stimulated; **, p<0.01 vs control EGF-stimulated, 2-way ANOVA.* **(D)** Concentration of NO_2^- in the media was measured using Griess assay. *n=4; *, p<0.05; ***, p<0.001 vs. untreated, One-way ANOVA.*

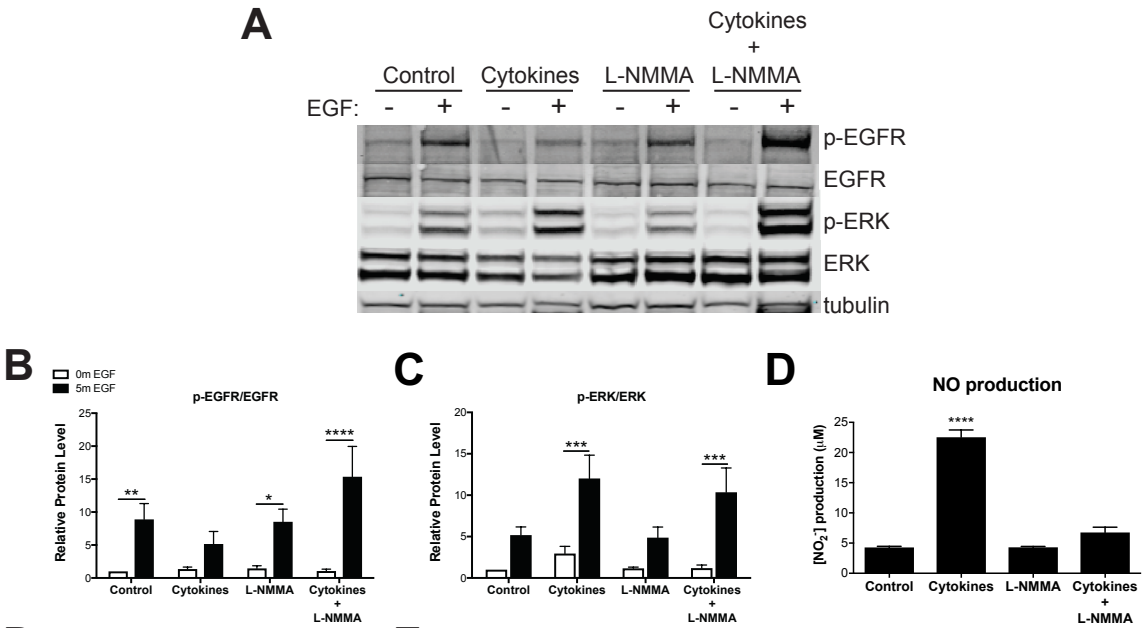


Figure 4-2. iNOS inhibition restores EGFR signaling. **(A)** Representative image of western blot where 832/13 cells were treated with cytokines (TNFα, IL-1β, IFN-γ) (16h) and/or iNOS inhibitor, L-NMMA (16h), starved for 2h in 2.5mM glucose and stimulated with 10 ng/ml rrEGF for 5 min. Cell lysates were collected for western blot analysis to determine p-EGFR, EGFR, p-ERK, ERK, and tubulin levels. **(B, C)** Quantified p-EGFR/EGFR and p-ERK/ERK shown as protein levels relative to non-treated groups and normalized to tubulin. *n = 7; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 vs non-EGF-stimulated, 2-way ANOVA.* **(D)** Concentration of NO_2^- in the media was measured using Griess assay. *n=3; ****, p<0.0001 vs. untreated, One-way ANOVA.*

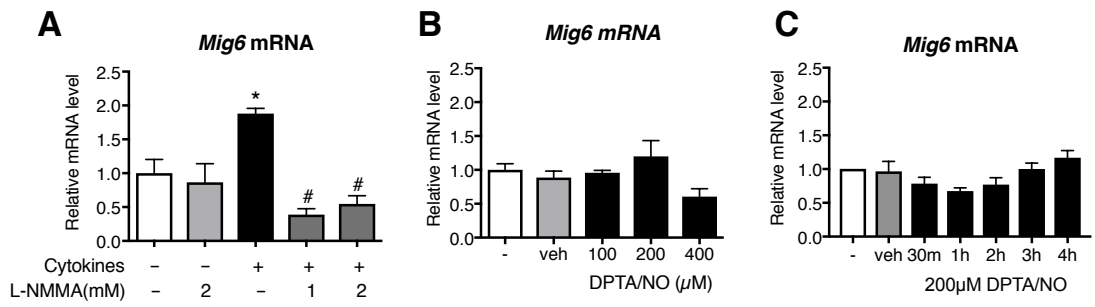


Figure 4-3. Cytokine-induced NO is necessary for Mig6 expression, but NO alone is insufficient. **(A)** 832/13 cells were treated with cytokines (TNFα, IL-1β, IFN-γ) and/or the iNOS inhibitor, L-NMMA for 24 h or **(B,C)** DPTA/NO at 100-400μM at times ranging from 30m to 4h**.** Cytokine effectiveness was confirmed by measuring *Nos2* mRNA (*data not shown*). *Mig6* and *Gapdh* mRNA levels were determined by qRT-PCR. *n = 3-6*; *, *p<0.05 vs. non-treated control, Student's t-test*; $^{\#}$, *p<0.001 vs. cytokine-treated, One-way ANOVA*.

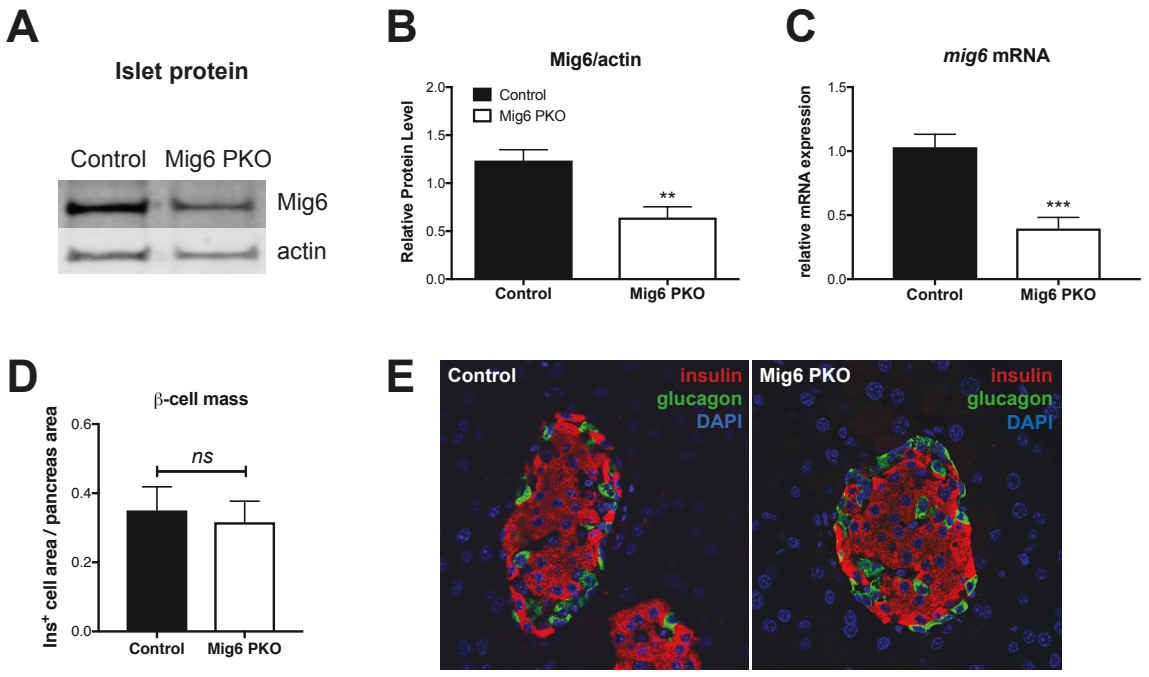


Figure 4-4. Pancreatic Mig6 knock out (Mig6 PKO) mice have decreased Mig6 protein levels and decreased mRNA expression. **(A)** Western blot representative image for graph **(B)** where islets were collected from control and Mig6 PKO mice and lysates collected for protein analysis. *n=6-8;* **, *p<0.01 Student's t-test.* Islets were isolated and cDNA was prepared by RT-PCR. **(C)** *Mig6* expression was determined by qPCR and normalized to *Gapdh* relative to a control sample. *n=6-10;* ***, *p<0.001 Student's t-test.* **(D)** Quantified islet area relative to total pancreatic area from saline-injected mice of both genotypes. *n=4-6.* **(E)** Images of immunofluorescent staining of insulin (red) and the glucagon (green), from an untreated control vs. Mig6 PKO mouse islet.

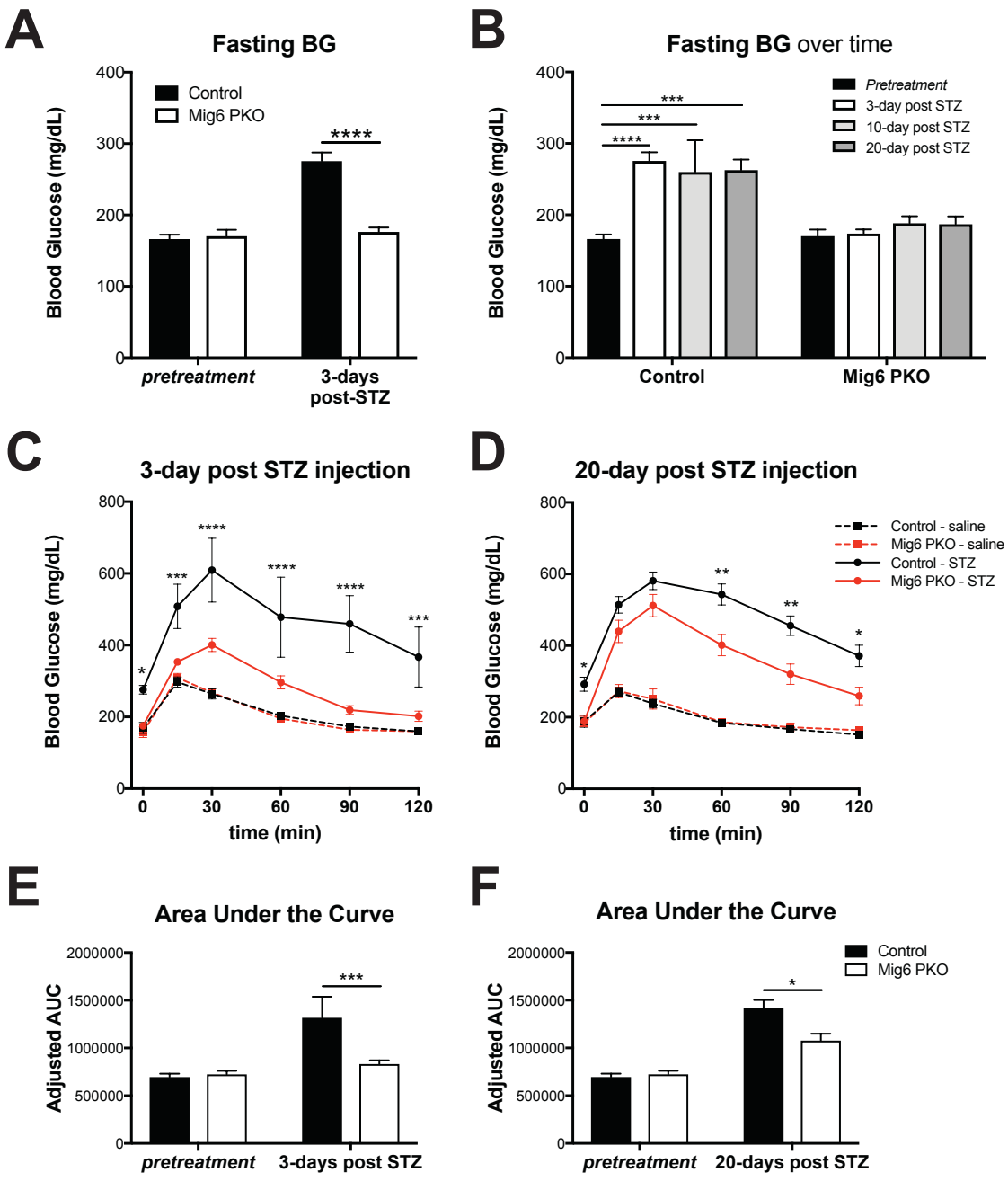

A
Fasting BG
Blood Glucose (mg/dL)
Control
Mig6 PKO
****
pretreatment
3-days post-STZ
B
Fasting BG over time
Blood Glucose (mg/dL)
Pretreatment
3-day post STZ
10-day post STZ
20-day post STZ
***
***
****
Control
Mig6 PKO
C
3-day post STZ injection
Blood Glucose (mg/dL)
time (min)
D
20-day post STZ injection
Blood Glucose (mg/dL)
Control - saline
Mig6 PKO - saline
Control - STZ
Mig6 PKO - STZ
time (min)
E
Area Under the Curve
Adjusted AUC
***
pretreatment
3-days post STZ
F
Area Under the Curve
Adjusted AUC
Control
Mig6 PKO
*
pretreatment
20-days post STZ

Figure 4-5. Mig6 PKO mice have a lower fasting blood glucose after STZ treatment, and have a slightly higher glucose tolerance than wild-type littermates. **(A)** 6-hour fasting blood glucose (FBG) of both genotypes before and after treatment. *n = 3 (STZ CTRL) n=16-24 (other groups)*; ****, *p<0.0001, 2-way ANOVA vs control.* **(B)** 6-hour FBG over time post-STZ treatment, showing that Mig6 PKO mice maintain FBG up to 20-days post STZ injection *n = 3 (STZ CTRL) n=16-24 (other groups)*; ***, *p<0.001;* ****, *p<0.0001 in 2-way ANOVA vs control pretreatment.* **(C)** 10-week old Mig6 PKO and control mice received daily injections of 35 mg/kg body weight of streptozotocin (STZ) or volumetric equivalent of isotonic saline over 5 consecutive days. Intraperitoneal glucose tolerance test (GTT) of Mig6 PKO and control mice 3 days and **(D)** 20 days post-STZ or post-saline injection. *, *p<0.05,* **, *p<0.01,* ***, *p<0.001,* ****, *p<0.0001 in multiple t-test vs Mig6PKO-STZ.* **(E)** Calculated area under the GTT curve at 3-days and **(F)** 20-days post STZ injection.

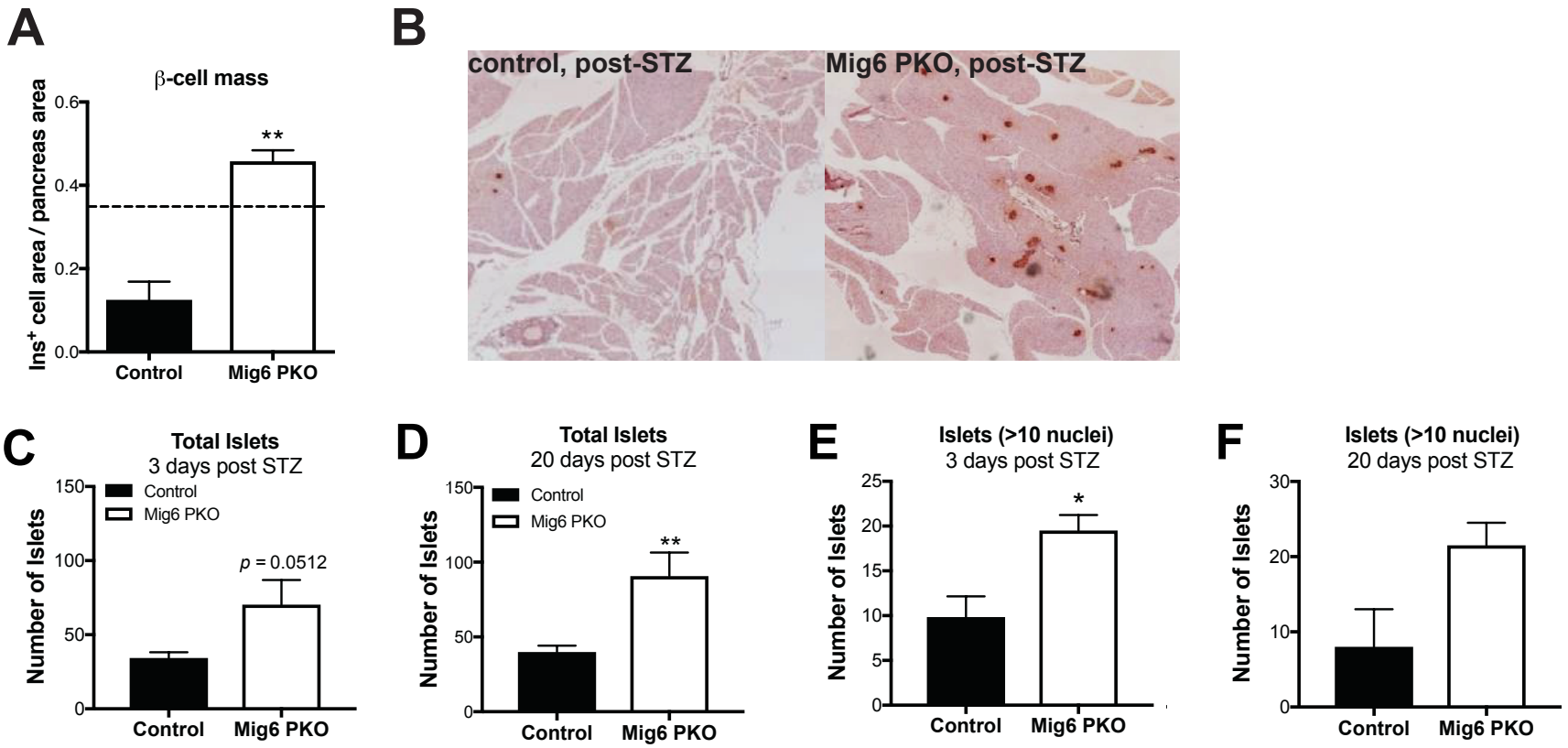

A
β-cell mass
Ins+ cell area / pancreas area
0.6
0.4
0.2
0.0
**
Control
Mig6 PKO
B
control, post-STZ
Mig6 PKO, post-STZ
C
Total Islets
3 days post STZ
Control
Mig6 PKO
Number of Islets
150
100
50
0
p = 0.0512
Control
Mig6 PKO
D
Total Islets
20 days post STZ
Control
Mig6 PKO
Number of Islets
150
100
50
0
**
Control
Mig6 PKO
E
Islets (>10 nuclei)
3 days post STZ
Number of Islets
25
20
15
10
5
0
*
Control
Mig6 PKO
F
Islets (>10 nuclei)
20 days post STZ
Number of Islets
30
20
10
0
Control
Mig6 PKO

Figure 4-6. Mig6 PKO mice have preserved β cell mass and preserved islet morphology. (**A)** Quantified islet area relative to total pancreatic area after STZ treatment (*dotted line represents saline-injected control mice*) calculated from **(B)** immunohistochemical staining for insulin in pancreatic sections of control and Mig6 PKO mice 20 days post-STZ treatment, *n = 4-6*; **, *p<0.01 vs control Student's t-test.* Immunofluorescent staining in pancreas sections of control and Mig6 PKO mice was employed to characterize islet morphology at 3- (C,E) and 20-days (G,I) post STZ injection and to calculate **(C,G)** total number of islets *n=3-5; **, p<0.01*, *Student's t-test, one-tailed,* **(E,I)** and islets containing more than 10 β cells. *n=3*; *, *p<0.05 Student's t-test, one-tailed.*

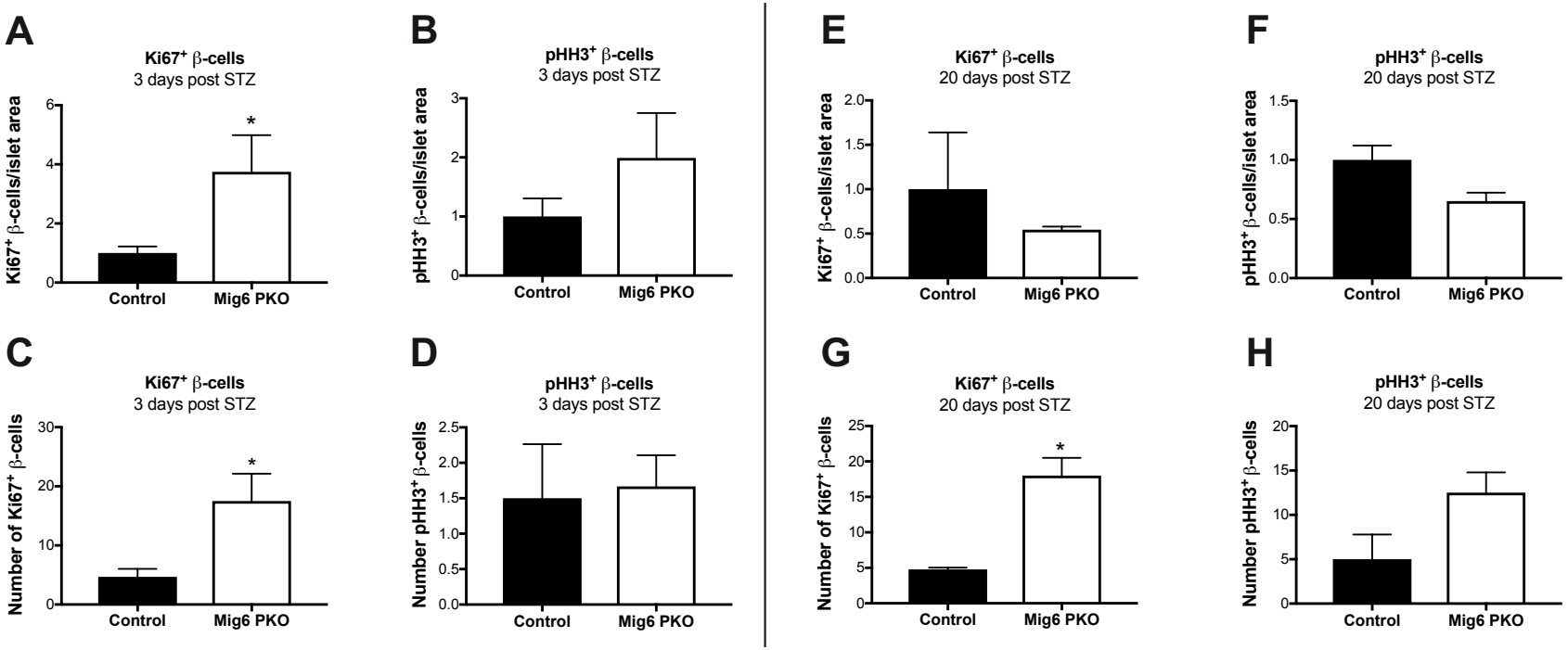


Figure 4-7. After STZ injury, Mig6 PKO mice initiate a rapid recovery response to increase β cell mass. **(A, C)** Ki67-positive β cells, detected by immunofluorescence, were counted and normalized to islet area at 3- and 20-days post STZ treatment. To detect specific cells committed to cell division, **(B, D)** phospho-histone H3 (pHH3)-positive β cells were counted and normalized to islet area. (Immunohistochemical staining for insulin in pancreatic sections of control and Mig6 PKO mice was used to calculate islet area). **(E, F, G, H)** Data graphed as absolute numbers of cells counted. *n=3 each group*; *, *p<0.05 Student's t-test, one-tailed.*

4.4 Discussion

EGFR signaling has been a focus of studies aiming to restore β cell mass in patients with diabetes. Past studies have illustrated the potential benefits of cellular therapies: combinatorial administration of EGF and gastrin [99] rescues hyperglycemia, and constitutively active EGFR have increased β cell mass [97]. In this study, we examined how pro-inflammatory cytokines, widely accepted to participate in the immune response during T1D, restrain EGFR signaling. Cytokines induce iNOS expression and NO production, and we further demonstrated that cytokine-induced NO is necessary for Mig6 expression. We also present evidence that EGFR signaling is dampened by NO, from cytokines or an NO donor itself. Given downstream ERK signaling was sometimes not changed in parallel to EGFR signaling, we redirect to reports that NO can trigger different effector mechanisms in the immune response depending on its concentration and ligand binding [233-236]. Therefore, we recognize that NO may have ancillary positive or negative effects on various tissues and cascades at varying concentrations. In 832/13 cells treated with NO donor, whereas EGFR signaling was dampened, ERK was not activated, suggesting that there are compensatory or secondary mediators of proliferation and survival in the β cell. Given the permissive role of NO to induce Mig6, there may be outcomes that are masked by a too broad use or saturated concentration of NO. Because the variable consequences and benefits of NO, it will be interesting to determine whether NO in variable concentrations or sources affects Mig6 expression and EGFR signaling.

We further refined our previous mouse model to a pancreas-specific knock out of Mig6 and we observed a comparable phenotype to our Mig6 haploinsufficient mouse

model. The increased glucose tolerance in the Mig6 PKO was significant but not as striking as the phenotype of our previous model. We include a measure of Mig6 knock down efficiency, which was about 50% knocked down. It is possible that a more complete knock down of Mig6 would result in an even more normal glucose tolerance after STZ β cell injury. Most notably, Mig6 PKO mice have normal fasting blood glucose which persists from 3- to 20- days post STZ treatment, with a more improved glucose tolerance at 3-days post STZ treatment, and this tolerance beginning to deteriorate approaching 20-days post injury. Mig6 PKO mice also have an improved β cell mass recovery after 20 days compared to their wildtype littermate control mice after STZ injury. Together, these data suggest that Mig6 accelerates the progression to diabetes after an immunological attack on the β cells.

Through histological analysis of pancreatic sections, we report that Mig6 PKO mice had increased numbers of Ki67-positive cells. Our previous report in the Mig6 haploinsufficient mouse model concluded that there is no difference in STZ-stimulated β cells that have entered into the M phase of the cell cycle. Here we report a more encompassing marker of proliferation, Ki67, which identifies all cells in any stage of the cell cycle. Because it is suggested that duct-associated islets and single extra-islet β cells indicate ongoing duct-to-islet neogenesis [237], we also noted the number of islets (including singlets) that were associated with a duct. Numbers of duct-associated islets were not different between the control and Mig6 PKO mice (*data not shown*), indicating an intrinsic change in cell fate. We defined the mechanism of β cell mass preservation as exploitation of β cell proliferation. The involvement and contribution of other means of β cell restoration, such as β cell transdifferentiation and neogenesis, which in other

transgenic models have been shown to potentially increase the reservoir of new β cells [195, 238], have yet to be published in the context of Mig6 knock down.

In summary, our data demonstrated that NO is detrimental to EGFR activation and phosphorylation, and is required for cytokine-mediated Mig6 expression. Yet NO alone does not induce Mig6. Like in our previous Mig6 haploinsufficient mouse model, when knocked down in the pancreas, Mig6 protects mice from chemically-induced hyperglycemia by preventing loss of β cell mass. This study highlights the pitfalls of treatments solely focused on the immunological response, as pathological stimuli can detrimentally impact mitogenic signaling and therefore EGFR regenerative mechanisms via Mig6 feedback inhibition. Mig6 presents a promising cellular therapeutic alternative that, when used to target β cell recovery programs, may prevent or reverse the progression to diabetes.

4.5 Materials & Methods

Animals and treatments

All animals were maintained and used in accordance with protocols approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee, following the *Guide for the care and use of laboratory animals*, Eighth edition (2011). Mice were maintained in a standard 12-hour light-dark cycle and provided unrestricted access to water and a standard rodent chow. *Tg*(*Pdx1-cre*) mice (Jackson Laboratory #014647) were bred with *Mig6*$^{flox/flox}$ or *Mig6*$^{flox/+}$ [230] on a C57Bl/6J background. The following primer sequences (Integrated DNA Technologies) were used to detect Pdx1-Cre and *Mig6*^{flox}: Pdx-forward = 5'-CTGGACTACATCTTGAGTTGC, Pdx-reverse = 5'-

GGTGTACGGTCAGTAAA-TTTG, *Mig6*^{flox}-forward = 5'-GGTCAGGGCTGTGCAGTCCGTAGA, *Mig6*^{Neo}-reverse = 5'-CGATACCCCACCGAGACC, and *Mig6*^{flox}-reverse = 5'-CTTCCCAAATCTAAC-ACCCGACAC. Extract-N-Amp Tissue PCR kit (Sigma-Aldrich, St Louis, MO, USA) was used for DNA extraction.

8 to 10-week old male mice of the genotypes Mig6$^{flox/flox}$/Cre^{+} (Mig6 pancreatic knock out), Mig6$^{flox/+}$/Cre^{-}, and Mig6$^{flox/flox}$/Cre^{-} (controls) were intraperitoneally injected with streptozotocin (STZ, 35mg/kg body weight; Sigma-Aldrich, St Louis, MO, USA) for 5 consecutive days as performed previously [105]. A group of control animals was injected in the same manner with vehicle (saline).

Metabolic tests

For glucose tolerance testing (GTT), 1.5 g/kg body weight D-glucose (Sigma-Aldrich) was intraperitoneally injected into 6-h-fasted control or STZ-treated mice. Blood was sampled from a tail vein at the indicated time points, and blood glucose was measured using an AlphaTrak glucometer (Abbott Laboratories, Abbott Park, IL, USA). Area under the curve (AUC) was calculated as the area under each point in the graph, averaged over the time between points, adjusting (subtracting) the initial fasting BG contribution.

Immunohistochemical and immunofluorescence staining

Immunostaining of pancreatic sections was performed as previously described [105]. Antibodies are listed in **Table 4-1.**

Table 4-1. Antibodies used for immunofluorescence staining

Name	Vendor, catalog number	Dilution
Anti-insulin	Santa Cruz, #H-86 Invitrogen, #180067	1:250 1:250
Anti-phospho histone H3	Millipore, #06-570	1:200
Anti-Ki67	Abcam, #15580	1:900

Islet experiments

Mouse islets were isolated and cultured as previously described [106, 220] for protein and mRNA analysis.

Cell experiments

INS-1-derived 832/13 rat insulinoma cells were cultured as previously described [221]. A starvation medium (RPMI 1640 containing 2.5 mmol/l glucose and 0.1% BSA) was used for EGF stimulation experiments. For cytokine/EGF stimulation experiments, 832/13 cells were pretreated with a pro-inflammatory rat cytokine 'cocktail' (1000 U/ml TNFα, 50 U/ml IL-1β, and 1000 U/ml IFN-γ; Prospec, East Brunswick, NJ) for 16 hours, starved for 2 hours and treated with 10 ng/ml rat recombinant EGF (R&D Systems, Minneapolis, MN, USA) for 5 min. For nitic oxide experiments, 832/13 cells were treated with L-NMMA (MilliporeSigma, St Louis, MO, USA) for 16 h, or DPTA/NO (Cayman Chemical, Ann Arbor, MI, USA) for 4 hours.

Immunoblot analysis

Immunoblot analysis was performed as previously described [106]. Phosphorylated protein levels were normalized to total protein levels, and total (i.e. non-phosphorylated) protein levels were normalized to tubulin or GAPDH protein levels. Antibodies are listed in **Table 4-2**.

Table 4-2. Antibodies used for immunoblotting

Name	Vendor, catalog number	Dilution	Solvent
anti-phospho-EGFR (Y1068)	Cell Signaling, #3777	1:1000	Nacalai USA Signal Enhancer Hakari 250
anti-EGFR	Sigma-Aldrich, #E3138	1:1000	Nacalai USA Signal Enhancer Hakari 250
anti-phospho-ERK1/2 (p42/44)	Cell Signaling, #4370	1:1000	PVP
anti-ERK1/2	Cell Signaling, #4696	1:1000	PVP
anti-γ-tubulin	Sigma-Aldrich, #T6557	1:2500	PVP
anti-Mig6	Santa Cruz, #D-1 21st Century Biosciences	1:250 1:200	PVP PVP

Quantitative RT-PCR analysis

RNA from 832/13 cells, and mouse, rat, and human islets was isolated using RNeasy Mini or Micro kits (Qiagen, Valencia, CA, USA). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan assays (Applied Biosystems) were used to obtain C_T values and the $\Delta\Delta C_T$ method was used to calculate the relative quantities of *mig6*, *gapdh*, or *NOS2* mRNA. Primer sequences were described previously [222]. PCR reactions were performed in triplicate for each sample from at least three independent experiments and normalized to *Gapdh* mRNA levels.

Statistical analyses

All data are presented as means ± SEM. Protein and mRNA data were normalized to control conditions and presented as relative expression. The Student's t-test (unpaired, two-tailed unless stated otherwise) or ANOVA (with Bonferroni post hoc tests) were performed using GraphPad Prism software (La Jolla, CA, USA) to detect statistical differences. $p < 0.05$ was considered statistically significant.

CHAPTER 5. DISCUSSION AND FUTURE STUDIES

Currently, autoimmune-mediated β cell ablation is irreversible and results in overt T1D. Cellular therapeutic strategies to increase β cell mass are a promising avenue to bypass current drawbacks of immunotherapies. Theoretically, targeted β cell expansion strategies together with re-education of immune cells to stop β cell destruction could be immensely successful in treating or even curing T1D. Yet, there are fundamental gaps in our understanding of both underlying mechanisms, thereby keeping this kind of therapy still very far away. EGFR has been strongly implicated in β cell preservation, but aberrant EGFR signaling is a deterrent for its use in clinical settings. Mig6 is a prime candidate for indirect EGFR signaling modification. We suggest that Mig6 has a role in both pancreas development and the progression to diabetes. From the data collected in this dissertation, I propose that Mig6 is a switch for duct progenitor cell differentiation. This notion is based on our findings in **Chapters 2 and 4**.

In **Chapter 2**, we demonstrated that a knock down in zebrafish *mig6* resulted in truncated exocrine pancreas development, fewer endocrine cells, and an enlarged EPD. These data, in conjunction with previous Mig6 studies in the developing embryo [181], suggest that there is a pool of duct progenitor cells that, normally, give rise to a variety of further differentiated cell types. Yet, without the actions of *mig6*, these progenitors may prematurely differentiate and become quiescent, reducing the number of endocrine cells by preferential differentiation of early pancreatic progenitor cells to non-endocrine duct cells. **Future studies** would confirm the developmental phenotype in CRISPR-generated

mutants, and attempt to rescue the *mig6MO* developmental phenotype with EGFR inhibitor injections.

Work in our lab has previously reported protection of STZ-induced β cell destruction in Mig6 heterozygous whole-body knock out mice [105]. We further refined our mouse model by creating a pancreas-specific Mig6 knock out mouse. Of course, between the tissue-specific Mig6 knock down in the mouse pancreas and the whole-body knock down of *mig6* in zebrafish, the Mig6 PKO model provides a more precise account of the function of Mig6 in the pancreas, as the zebrafish pancreas phenotype may be a secondary effect of *mig6* knock down in peripheral tissues such as liver or heart. In any case, whole-body knock down of Mig6 is probably not a translatable therapy to humans, as homozygous Mig6 knock out mice readily develop tumors [239-241]. More likely, targeting tissue-specific or cell-specific changes in Mig6 would be a more practical means to induce β cell regeneration. Efforts to develop targeted therapeutics continue to develop rapidly [242].

I speculate that Mig6 PKO mice have intact ductal progenitor cells due to incomplete knock down of Mig6. In **Chapter 4**, we observed increased β cell proliferation in response to STZ treatment in the Mig6 PKO mice. If the effects of Mig6 on progenitor cell differentiation were unnoticed due to insufficient knock down, then integration of our findings in **Chapter 2** leads us to hypothesize that a significant progenitor pool in the mouse pancreatic ducts was not altered and remained available for cell specification when subjected to β cell toxins. Contributing to this hypothesis is the observed increase in ventral bud-derived β cells in the zebrafish, which could signify an increased rate of proliferation or transdifferentiation in response to insufficient β cell

differentiation from pancreas progenitor cells. It is necessary to bring attention to the trending reduction (although not significant) in dorsal bud-derived β cells in the generation of this hypothesis.

Future studies will elaborate on these findings by using a heat-shock inducible Cas9 to generate an inducible *mig6* CRISPR mutant, to study the effect of *mig6* without disrupting pancreas development. Using this model, we may see a similar increase in β cell regeneration in *mig6* knock down as we saw in the Mig6 PKO mice.

At first glance, our results in **Chapter 4** would indicate a different role for mouse Mig6 in endocrine cell fate. We did not observe a change in β cell mass between control and Mig6 knock out mice as we did in the zebrafish. It is reasonable to assume because of the knock down efficiency in the mouse, there was functional Mig6 protein during key stages of development whose effects were uncovered only in the presence of β cell injury. If we assume our initial hypothesis from **Chapter 3** is accurate, that Src kinase activation results in increased p-Mig6, we might gather that the rise in EGFR signaling observed in Figure 3-4 C was a result of decreased Mig6 activity, and this was only detected under cell stress conditions. This result would complement the β cell mass preservation in our Mig6 PKO mice treated with STZ.

In **Chapter 3**, we investigated the facilitative effects of Src kinase, a possible regulator of Mig6 activity. We recapitulated, in the 832/13 cells and in isolated mouse islets, that Src kinase was activated under treatments with high glucose or cytokines. Further studies and alternative techniques are required to answer the questions posed in Chapter 3. Whereas all of the experiments in Chapter 3 focused on Src inhibition, Src overexpression would complement and perhaps enhance the data. However, because

overexpression of native Src itself may not correlate with increased activated Src, we would be relying on cytokine treatment and EGF stimulation to induce stoichiometric increases in phosphorylated Src. Instead transducing 832/13 cells with Ad-CMV-Src529, a constitutively active form of Src, would be more informative. Src529 is an Y529F mutant that prevents the normal downregulation of kinase activity and thus results in unregulated kinase activity of Src [243].

Taken together, these data implicate Mig6 in at least two approaches to β cell restoration. First, Mig6 is required for proper differentiation of duct progenitor cells into endocrine cells, a mechanism with significant potential in β cell regeneration strategies (like the creation of stem cell-derived β-like cells or increasing endogenous β cell regeneration potential). And second, Mig6 blocks β cell proliferation through inhibition of EGFR-mediated recovery programs. Targeted ablation of Mig6 in the pancreas could sufficiently increase β cell mass during the honeymoon phase. Thus, Mig6 should be considered a prominent candidate for the treatment of diabetes. Not only could Mig6 command pancreatic differentiation to generate β-like cells (*in vivo*, or *in vitro*), but Mig6 also presents a promising cellular therapeutic alternative that, when used to target β cell recovery programs like EGFR, may restore β cell mass and reverse diabetes.

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CURRICULUM VITAE

Kimberley Mei Ling El

EDUCATION

Indiana University, Indianapolis, IN
August 2013 – October 2018
Ph.D., Cellular & Integrative Physiology, Diabetes minor
Dissertation: The Role of Mig6 in Pancreas Development and Diabetes.
Advisor: Patrick T. Fueger, Ph.D.

Indiana University Kelley School of Business, Indianapolis, IN
August 2017 – May 2018
Certificate, Business in Life Sciences

Hanover College, Hanover, IN
September 2009 – May 2013
B.A., Chemistry, Biology minor
Thesis: Determination of resonance energy in a benzene ring by oxygen bomb calorimetry of n-hexylbenzene, trans, trans, cis-1,5,9-cyclododecatriene and trans, trans, cis-2,6,10-dodecatriene.
Advisor: Steve C. Boone, Ph.D.

GRANTS & AWARDS

Pre-Doctoral Fellowship
American Heart Association
July 2016 – July 2018

Research Recognition Award
APS Endocrinology & Metabolism Section
April 2016

Oral Presentation Award
IUSM Sigma Xi Research Competition, 3rd place
November 2016

RESEARCH EXPERIENCE

Indiana University School of Medicine
May 2014 – September 2018
Advisor: Patrick T. Fueger, PhD
Projects:

- Characterized and proposed a mechanism of β cell regeneration in a pancreas-specific mitogen-inducible gene 6 (Mig6) knock out mouse model.

- Defined the role of *mig6* in pancreas development in zebrafish, a collaborative investigation that described *mig6* as a novel regulator of pancreas and liver development in zebrafish.
- Investigated the regulation of Mig6 by Src kinase during the progression to diabetes.

Hanover College
August 2012 – May 2013
Advisor: Steve C. Boone, PhD
Project: Determined the resonance energy of a benzene ring.

PUBLICATIONS

1. **KML El**, PT Fueger, RM Anderson. "*Mig6* is Required for Pancreas Development" 2018 (*in progress*)

2. **KML El**, Y-C Chen, B Bauer, A Rezaeizadeh, PT Fueger. "Mig6 Accelerates the Progression to Diabetes by Inhibiting EGFR-Mediated Regeneration Mechanisms" 2018 (*in progress*)

3. Y-C Chen, **KML Fong**, PT Fueger. "Extending the honeymoon: Harnessing the β cell's endogenous ability to regenerate." *Journal of Investigative Medicine* 2017 (*submitted, invited review*)

TEACHING & PRESENTATIONS

Oral Presentations

1. **KML El**. "Endocrine Pancreas," and "Diabetes" lectures IU School of Medicine Guest Lecturer, Graduate Level Physiology. Indianapolis, IN. (2017)

2. **KML Fong**, PT Fueger. "Mitogen-inducible Gene 6 Accelerates the Progression to Diabetes by Inhibiting EGFR-mediated Repair Mechanisms." Midwest Islet Club. Indianapolis, IN. (2016)

Poster Presentations

3. **KML El**, PT Fueger, RM Anderson. "*Mig6* is Required for Exocrine Pancreas Development in Zebrafish." American Diabetes Association. Orlando, FL. (2018)

4. **KML El**, PT Fueger, RM Anderson. "*Mig6* is Required for Exocrine Pancreas Development in Zebrafish." Indiana Physiological Society. Upland, IN. (2018)

5. **KML Fong**, A Rezaeizadeh, YC Chen, PT Fueger. "Cytokines Activate Src Kinase and Inhibit EGFR-Mediated Repair Mechanisms via Mig6 in Pancreatic β Cells." Experimental Biology**.** Chicago, IL. (2017)

6. **KML Fong**, PT Fueger. "Mitogen-inducible Gene 6 Accelerates the Progression to Diabetes by Inhibiting EGFR-mediated Repair Mechanisms." Experimental Biology San Diego, CA. (2016)

7. **KML Fong**, PT Fueger. "Mitogen-inducible Gene 6 Accelerates the Progression to Diabetes by Inhibiting EGFR-mediated Repair Mechanisms" Indiana Physiological Society. Greencastle, IN. (2016)

8. **KML Fong**, Y-C Chen, PT Fueger. "Loss of Pancreatic Mitogen-Inducible Gene 6 Protects Against Streptozotocin-induced Diabetes." Experimental Biology Boston, MA. (2015)

9. **KML Fong**, Y-C Chen, PT Fueger. "Loss of Pancreatic Mitogen-Inducible Gene 6 Protects Against Streptozotocin-induced Diabetes." Indiana Physiological Society Indianapolis, IN. (2015)

RESEARCH COMMITTEE

Co-Chair: **Patrick T. Fueger**, **Ph.D.**, Associate Professor
Beckman Research Institute, City of Hope, CA
Department of Molecular and Cellular Endocrinology

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Indiana University School of Medicine, Indianapolis, IN
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Ryan M. Anderson, **Ph.D.**, Assistant Professor
Indiana University School of Medicine, Indianapolis, IN
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Indiana University School of Medicine, Indianapolis, IN
Department of Biochemistry and Molecular Biology

Laura Haneline, M.D., Professor
Indiana University School of Medicine, Indianapolis, IN
Department of Cellular & Integrative Physiology

LEADERSHIP & COMMUNITY ENGAGEMENT

Molecular Medicine in Action
Indiana University Purdue University Indianapolis
Indianapolis, IN
2014 – 2018
Diabetes Supermodule Workstation Assistant

IUSM Biomedical Gateway Program
Indiana University Purdue University Indianapolis
Indianapolis, IN
2015 – 2018
Graduate Student Mentor

PROFESSIONAL ORGANIZATIONS

American Physiological Society, 2014 – 2018
American Heart Association, 2015 – 2018
Indiana Physiological Society, 2014 – 2016
SACNAS, IUPUI Chapter, Secretary (2014), 2014 – 2015